MOLECULAR BIOLOGY OF CAULIFLOWER MOSAIC VIRUS -

a potential genetic vector.

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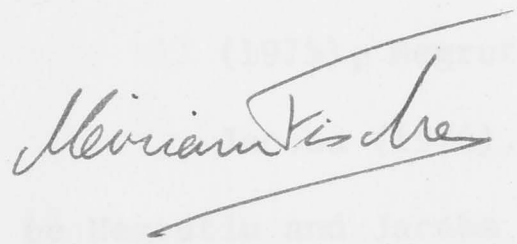
TO MY MOTHER

AND

IN MEMORY OF MY FATHER

DECLARATION

All the work reported in this thesis was done by myself except for the "cutting" and "ligation" of DNA molecules described in Chapter 3 (Section 3.5.9) which was done by Dr. J. Langridge, and where due reference is made in the text. No part of the work described has been reported elsewhere for the award of any other degree or diploma.



(Miriam Fischer)

LIST OF CORRECTIONS

Page No.	Line	Error	Correction
2	22	Ref. 'Arber *et al.*'	should be Arber and Dussoix
2	24	Ref. 'Smith *et al.*'	should be Smith and Wilcox
99	3	Negrutiu (1975, 1976)	should be Negrutiu *et al.* (1975), Negrutiu and Jacobs (1976).
101	4	Negrutiu *et al.* (1975, 1976)	should be Negrutiu *et al.* (1975), Negrutiu and Jacobs (1976).
109	18	Negrutiu, 1976)	should be Negrutiu and Jacobs (1976).

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List of Abbreviations

A = adenine

A_{260} = optical absorbance at 260 nm

A_{280} = optical absorbance at 280 nm

ATP = adenosine triphosphate

ATP^{32} = [α-^{32}P]-adenosine 5'-triphosphate

B_5 = Tissue culture media (Gamborg *et al.*, 1968).

BAP = 6 benzylamino-purine

C = cytosine

CaMV = Cauliflower mosaic virus.

Ci = Curie

c-RNA = ribonucleic acid synthesised from a double stranded DNA template.

CS 5 = Tissue culture media (Gibson *et al.*, 1976)

CSIRO = Commonwealth Scientific and Industrial Research Organization.

CTP^{32} = [α-^{32}P]-cytidine 5'-triphosphate

cv. = cultivar

dATP = 2'-deoxy adenosine 5'-triphosphate

dCTP = 2'deoxy cytidine 5'-triphosphate

DTT = dithiothreitol

dGTP = 2'-deoxyguanosine 5'-triphosphate

DNA = deoxyribonucleic acid

dTTP = 2-deoxythymidine 5'-triphosphate

2,4-D = 2,4-dichlorophenoxyacetic acid

2,4,5-T = 2,4,5-trichlorophenoxyacetic acid

2iP = N^6-(Δ^2-isopentenyl)-adenine

EDTA = [ethylene diamine]-tetra acetic acid, disodium salt.

G = guanine

GTP^{32} = [α-^{32}P]-guanosine 5'triphosphate

IBA = indole butyric acid

kinetin = 6-furfurylaminopurine

M&S = Murashige and Skoog media (1962)

mA = milliamps

mRNA = messenger ribonucleic acid

OD = optical density

pCPA = para-chlorophenoxyacetic acid

^{32}P cRNA = radioactively labelled ribonucleic acid obtained from a double-stranded DNA template.

POPOP = 1,4-Bis[2-(4-methyl-5-phenyloxazolyl)]-benzene.

PPO = 2,5-Diphenyloxazole

RNA = ribonucleic acid

rpm = revolutions per minute

SDS = sodium dodecyl sulfate

SSC = standard saline-sodium citrate buffer

T = thymine

TE buffer = 10 mM Tris-1 mM EDTA buffer pH 8.4

TES buffer = 30 mM Tris-2.5 mM EDTA-50 mM NaCl buffer pH 8.0

Tm = Temperature at which 50% of double stranded nucleic acid has denatured.

Tris = tris-hydroxymethyl aminomethane

U.V. = ultraviolet light

UTP^{32} = [α-^{32}P]-uridine 5'triphosphate

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ABSTRACT

Cauliflower Mosaic Virus (CaMV), which is a plant virus whose genome is double-stranded DNA, could perhaps be used as a vector for 'transformation' of plants. This thesis reports experiments which examine several aspects of the organization of the CaMV genome, experiments on the infectivity of intact and modified CaMV genomes using a reliable local lesion assay developed to assess infectivity, and experiments on cultured cells of potential plant hosts.

The genome organizations of three isolates of CaMV (New York 8135, Campbell and A.C.T.) were examined. A restriction endonuclease map of CaMV genomic DNA for each of the strains was constructed using Eco Rl, Sal I, Bam I, Xho I, Xba I and Hind III. The positions of the restriction sites were assessed by measuring the sizes and number of fragments produced when the CaMV genome was (a) digested with a particular enzyme or with that enzyme and a second enzyme, which was usually Eco Rl; (b) partially digested by a particular enzyme; and (c) digested with an enzyme and then a specific fragment isolated and then digested by a second restriction endonuclease. This latter procedure was used to confirm the fragment positions determined by (a) and (b).

Two of the six enzymes produced specific fragments which differentiated between the DNA of the three strains. The strain New York lacked one Eco Rl restriction site which was present in both Campbell and A.C.T., and the strain A.C.T. contained different Hind III sites from those in Campbell or New York. Mapping was made difficult by the varying proportion of linear and circular DNA molecules in different preparations. Heterogeneity within strains was also apparent, in the form of cleavage sites present in only a small

proportion of the native DNA molecule.

The DNA of each of the three strains contained three single-stranded gaps; one in one strand and two in the complementary strand. The position of the gaps with respect to the restriction endonuclease map was determined and their nature examined using different procedures; gel electrophoresis in neutral and alkaline conditions, hybridization kinetics and electron microscopy. The hybridization studies indicated that sequences within the CaMV genome are repeated.

A possible association between the presence of single-stranded gaps and infectivity was studied by comparing the infectivity of cloned CaMV DNA, in which gaps are absent, with native CaMV DNA. Cloned DNA was not infective, but the loss of infectivity could also be due to the restriction site used to clone the genome. Attempts were made to determine the position of ribonucleotides present within the CaMV genome, that might serve as primers of replication.

Several systems of protoplast-cell tissue culture in which CaMV could multiply were studied. Cell cultures of several species which are hosts for CaMV were obtained. The best cultures were obtained from rapeseed cv. Turret, *Nicotiana clevelandii* and *Arabidopsis thaliana*. Of these, suspension cultures were successfully established for *N. clevelandii* and *A. thaliana*. Protoplasts were isolated from numerous *Brassica* sp. but they could not be cultured.

The exonuclease and endonuclease content of plant extracts and commercial enzymes used in the production of protoplasts was examined. A monitoring system to evaluate nuclease activity was developed.

The results were used to assess the potential of CaMV as a vector of genetic information.

CHAPTER 1.

INTRODUCTION

1.1. GENERAL INTRODUCTION

In 1945 the United Nations Organization recognized freedom from starvation as a basic human right to be secured for all people. Together with achieving a more equitable distribution of food resources, increasing food production is one of the most pressing problems of this era. An increase in production of 2.5% per year is necessary just to maintain the present level of nutrition and an increase of an additional 1.0% would be required in order to mitigate malnutrition in a large percentage of the world population (World Food and Nutrition Study, 1975).

One important way in which crop production has been increased has been by genetic improvement of crop varieties. Doubtless this will continue to be important in the foreseeable future. However, to meet the increasing demand for food and energy conservation, new and novel genetic techniques will be required by agriculture to increase production of protein of better nutritional quality and to wage the inevitable battle against insects and pathogenic diseases. Mutagenesis, polyploidization and the use of population genetic concepts have each become part of the plant breeder's armoury, and it now seems likely that advances in the field of molecular genetics and plant cell culture will provide technology to further improve crop plants.

This technology holds the promise that barriers to gene flow between species can be circumvented to provide new and increased levels of genetic variation and that desirable gene combinations can be more efficiently identified.

The various technologies include plant somatic cell genetics, protoplast isolation and culture, somatic hybridization, production of haploids by anther culture and recombinant DNA research (Scowcroft, 1977).

The investigation reported in this thesis was primarily aimed at recombinant DNA research in conjunction with plant cell culture.

1.2. RECOMBINANT DNA TECHNOLOGY

The discovery and exploitation of two natural phenomena found in bacteria has opened new possibilities of altering the genetic make-up of a species.

The first discovery was that of plasmids, which are autonomous, stable, extrachromosomal genetic elements, some of which may be able to reversibly integrate with the "host" chromosome.

Plasmids carry non-metabolically essential genes, such as those conferring drug or heavy metal resistance, the production of antibiotics or the ability to metabolize long chain hydrocarbons. The main functions conferred by plasmids allow their "hosts" to adapt to new environments. Some plasmids also promote bacterial conjugation, and in this way allow the spread not only of plasmid genes but also they make genetic recombination possible with the "host" chromosome.

The second discovery was that of restriction endonucleases whose presence was initially predicted by Arber *et al.* (1962). Almost a decade elapsed before these were isolated and characterized by Smith *et al.* (1970) and Danna and Nathans (1971) who showed that these enzymes could be used for *in vitro* genetic manipulation (Smith, 1979).

These enzymes catalyze the cleavage of DNA molecules at specific sites regardless of its origin. Some of the enzymes cleave double-

stranded DNA at particular palindromic sequences and produce symmetrical 'staggered cuts'. The single-stranded ends of such DNA fragments have a nucleotide sequence which is complementary with that of any other DNA fragment cut by the same restriction endonuclease.

These two discoveries opened new possibilities for manipulating genes.

In plants the manipulation of genes has been called "transformation" by Holl *et al.* (1974) since it allows genetic information to be transferred across the natural breeding barrier between plant taxa.

1.3. PLANT TRANSFORMATION: GENETIC MANIPULATION BY UNCONVENTIONAL MEANS

The stages in this process can be discussed under the following headings.

(a) Sources of novel genetic material. The recognition, characterization and isolation of the gene or genes which one desires to transfer to other species.

(b) The techniques by which the genetic material is transferred between species.

(c) Integration, replication and subsequent expression of the novel genes in the new "host".

1.3.1. *Sources of novel genetic material*

The techniques of cloning DNA molecules allow specific fragments of DNA from procaryotic or eucaryotic organisms to be recombined *in vitro* with a procaryotic plasmid or virus, which will act as its vector and take it into a bacterial cell where it will be replicated (Helinski, 1977). Most commonly, plasmids or phages are used as vectors and *E. coli* as the "host" procaryote (Helinski, 1977).

This technique permits one to amplify and isolate large amounts of specific DNA fragments free of other chromosomal DNA of the donor. Moreover, cloning techniques are now so sophisticated that the entire haploid genome of any eucaryote can be efficiently cloned into a 'library' of DNA fragments (Maniatis *et al.*, 1978).

However, it is not merely sufficient to clone the entire genome. Before such cloned fragments can be meaningfully used, it is essential to correlate one or a few specific fragments with a specific gene function. Where an adequate probe, such as m-RNA is available, the identification of the corresponding cloned DNA fragment is relatively straight forward. Alternatively, specific fragments can be isolated where a gene function can be effectively selected in a bacterial host such as in the case of the dihydrofolate reductase (DHFR) from rat pancreas selected in DHFR$^-$ *E. coli* (Chang *et al.*, 1978).

These molecular cloning techniques have had a major impact on understanding the organization and function of genes in eucaryotes although caution should be exercised in planning to use these concepts to improve crop plants. For instance, in traits which are known to be polygenically inherited, e.g., yield, maturity, photosynthetic efficiency, it seems unlikely that one could even identify, let alone assemble, all the essential genes involved. Characteristics which have a less complicated physiological and biochemical basis may be more amenable to manipulation. In this category might be included traits such as resistance to specific pests, diseases, environmental conditions such as drought, heavy soils, high salinity, acidity, resistance to pathotoxins and the ability to fix nitrogen. The way in which each trait is expressed and interacts, should also be well understood and this is made easier by the technique of DNA cloning,

which is being extensively used in examining how known genes function in procaryotes and eucaryotes.

1.3.2. *Recipient plant material. Transfer of genetic material between species*

The uptake of DNA by plants has been shown using a number of different techniques and combinations of DNA donor and acceptor (Kleinhofs and Behki, 1977). There seems to be a general agreement that exogenous DNA is taken up by seeds, germinating embryos, by excised seedlings and especially by protoplasts.

Protoplasts, plant cells whose cellular walls have been removed by mechanical or enzymatic treatments, readily absorb macromolecules, including DNA.

While mechanical methods of isolating protoplasts have been known for decades, Cocking (1960), using an impure enzyme derived from *Myrothecium verrucaria* demonstrated that protoplasts from tomato roots could be isolated in large numbers. Since then protoplast technology has developed very rapidly; the isolation, culture and uses of plant protoplasts have been extensively reviewed (Cocking, 1972; Takebe, 1975; Vasil, 1976; Gamborg and Wetter, 1975).

Protoplasts can be isolated from a wide variety of tissues and organs and from virtually any plant structure that is not lignified, but those from mesophyll tissue of leaves have proved most suitable for culture and further growth (Vasil, 1976). Protoplasts have also been isolated from callus cells of different species and from suspension cultures (Vasil, 1976). These sources can be more readily standarized as they can be grown in controlled conditions and known nutrients.

Plant protoplasts appear to be ideal for experimental plant improvement because, in theory, each cell is totipotent, also because they can be induced to fuse readily with each other (Power *et al.*, 1970; Cocking, 1975), and because they can absorb macromolecules such as virus particles (Takebe and Otsuki, 1969; Aoki and Takebe, 1969), polystyrene latex particles (Mayo and Cocking, 1969), bacteria (Davey and Cocking, 1972), chloroplast (Bonnet and Erikson, 1974) and especially DNA.

The absorption of DNA will be discussed in more detail.

1.3.3. *DNA Absorption*

Holl *et al.* (1974) in their review concluded that Ledoux *et al.* in their experiments with germinating barley, tomato plants and seeds of *Arabidopsis thaliana* had successfully proved the absorption of DNA.

The successful uptake of DNA by protoplasts of different species has been reported repeatedly. Hess (1970, 1975) showed that *Petunia* protoplasts absorbed *Petunia* DNA. Ohyama *et al.* (1972) found that protoplasts of *Ammi visnaga* absorbed *E. coli* DNA, and Suzuki and Takebe (1976, 1978) have studied the optimal conditions for absorbtion by tobacco protoplasts of single-stranded DNA genome of bacteriophage fd. and the double-stranded DNA genome of bacteriophage λ.

Hughes *et al.* (1978) using barley and tobacco leaf protoplasts demonstrated that uptake of bacterial DNA (*Bacillus subtilis*) was generally a linear function of both time (0-6 hrs) and DNA concentration (0-200 µg/ml). They also found that nuclei, isolated after uptake, contained 60-80% of the absorbed DNA but that 80% of it was degraded after 4 hrs.

Ohyama (1978) assessed the amount of DNA absorbed and bound by nuclei isolated from *Glycine max* L. Merr. protoplasts, of the single-stranded genome DNA of bacteriophage fd. and double-stranded genome DNA of *Salmonella typhimurium*.

1.3.4. *Integration, replication and expression of exogenous DNA*

The fate of DNA absorbed by plants is a very controversial subject. Of 50 experimental papers published on the matter, about one quarter are concerned with attempts, usually unsuccessful, to reproduce experiments published by others (Langridge, 1978).

Kleinhofs and Behki (1977) have reviewed the evidence reported for DNA uptake by plant cells and its subsequent integration and replication in the host genome, and concluded that up to that date, no unequivocal proofs of integration and replication had been achieved.

Plasmids (Lurquin and Kado, 1977; Fernandez *et al.*, 1978) and other forms of DNA (Hughes *et al.*, 1978) have been shown to be absorbed by plants but a very small percentage (1-3%) of that absorbed is retained as macromolecules, most is degraded. For example 97% of the plasmid pBR313 absorbed by cowpea protoplast (Fernandez *et al.*, 1978) was hydrolyzed into nucleotides, the remaining 3% was cleaved, into an average of 2-3 polynucleotide chains, although in turnip protoplast, the resistant 1% of molecules remained intact for 21 hours. Lurquin and Kado (1977) also showed that the absorbed DNA became associated with the nucleus but that DNA was not integrated into the chromosomal DNA.

The evidence for expression of genes introduced into plant cells as purified DNA is also very inconclusive (Scowcroft, 1977) and the expression, if present, may only be transitory. Carlson (1973) infected barley protoplasts with a bacteriophage of *E. coli* and was able to

detect two phage-specific enzymes, a S-adenosylmethionine cleaving enzyme and RNA polymerase. The expression of these functions was rapid and transitory. Hess (1975) also had similar transitory expression of foreign DNA working with Petunia and Merriam and Scowcroft (personal communication) also had some success in growing kanamycin sensitive tobacco cells in a medium containing kanamycin after the cells were treated with plasmid DNA from *E. coli* which carried kanamycin resistance. But the results did not unequivocally show that the exogenous DNA had been integrated and expressed.

The selection and/or construction of vectors may influence the successful expression of inserted genes. In procaryotes, genes transferred to procaryotes by vectors such as plasmids usually function (Meagher, 1977). In lower eucaryotes, such as the yeast DNA fragment coding for a histidine biosynthetic enzyme, imidazole glycerol-phosphate dehydratase, tryptophan synthetase, and *Neurospora crassa* DNA coding for the catabolic dehydroquinase (Meagher, 1977), are functionally expressed when introduced into *E. coli* and are capable of complementing auxotrophic mutants for these enzymes. But DNA from higher eucaryotes contained in recombinant clones seems not always to be correctly expressed in bacteria, such as *E. coli*. For example, when mouse mitochondrial DNA was cloned in the pSC101 plasmid of *E. coli* (Chang *et al.*, 1975), four possible configurations of chimaeric molecules containing the mitochondrial DNA were identified. But the RNA synthesized in *E. coli* minicells was found to be predominantly transcribed from the light strand of the mitochondrial DNA, implying that initiation of RNA synthesis occurs within the mitochondrial DNA fragment. Although specific polypeptide synthesis was directed by the mitochondrial DNA, the polypeptides synthesized consisted primarily

of small molecules different from those observed in the mitochondria of mouse L cells.

Similarly, fragments of sea urchin histone genes, and *Drosophila melanogaster* DNA contained in recombinant vectors have been transcribed, but in general this transcription has been incorrect (Meagher, 1977).

This might suggest that it will be necessary to develop different molecular cloning systems for higher organisms. It may be essential to use as vectors genomes that are already able to replicate in the higher organisms.

Langridge (1978) suggested that the main deficiency in the plant transformation experiments already reported was that people had not used transforming molecules which could be expected to replicate in the plant cells or to become integrated into the plant DNA. He suggested that an ideal molecule for transformation would be a double-stranded DNA, circular in form as this would be able to replicate and to be integrated. The construction of this vector would require the isolation and joining together of five DNA sequences.

(a) A sequence recognized by the DNA polymerase of the bacterial cell in which the vector was constructed.

(b) A sequence recognized by the DNA polymerase of the plant cell to allow replication in the plant.

(c) Two genes, whose phenotypic expression could be used for the selection of bacterial and plant cells, respectively.

(d) A sequence of host plant DNA that would promote recombination.

(e) One intact restriction endonuclease site which could be used for the insertion of other foreign plant genes.

Within the plant kingdom the number of replication sequences adapted to multiply in plants is rather limited, (see below), and this narrows the chances of finding an appropriate vector.

1.3.4.1. Molecular vectors that can be used for gene transfer in plants

Currently the limited number of DNA molecules which can be used for vector construction, i.e., contain a "plant" DNA polymerase include:

(a) The DNA of plant organelles.

Chloroplast DNA with a molecular weight of 90-100 x 10^6 and mitochondrial DNA molecular weight 40-50 x 10^6 (Scowcroft, 1977). However the replication sequences of these DNAs have not been isolated.

(b) The 2 µm plasmid of yeast.

Several strains of yeast, *Saccharomyces cerevisiae*, contain a circular double-stranded plasmid of approximately 2 µm in length (Hollenberg *et al.*, 1976; Cameron *et al.*, 1977). This plasmid has been used successfully as a self-replicating vector to transform yeast cells (Gerbaud *et al.*, 1979), although integration of the vector in the nuclear DNA has not been proved.

The transformation of yeast can make possible the cloning of eucaryotic genes into a eucaryotic host, but still leaves unsolved the problem of transferring genetic information to higher plants.

(c) The replicon of the tumour inducing plasmid of *Agrobacterium tumefaciens*

Evidence (Watson *et al.*, 1975) shows that a large plasmid, the Ti plasmid, is involved in the production of tumours by this

bacterium in a large number of hosts. There is also evidence that at least a part of this plasmid is present in the DNA of bacteria-free tumour cell cultures (Chilton *et al.*, 1977) and is transcribed in these cells (Drummond *et al.*, 1977). This is perhaps the first evidence that DNA of bacterial origin has been transferred from bacteria to plants and it might be possible to use this plasmid to introduce foreign genetic information into plant cells. Experiments using this plasmid however have generally been restricted by biosafety regulations for work with recombinant DNA.

(d) Plant viruses with a DNA genome.

The genome of some plant viruses consists of DNA. This DNA is, of course, capable of autonomous replication in plant cells and possibly could be used as a vector for exogenous DNA.

Among the plant viruses there are only two distinctive taxonomic groups that have DNA as their genomes.

One group, is the caulimoviruses (Harrison *et al.*, 1971) of which cauliflower mosaic virus is the best known member and it will be reviewed in detail in Chapter 2. Several other viruses, such as, dahlia mosaic, carnation etched ring viruses, figwort mosaic virus, mirabilis mosaic, strawberry vein banding and probably cassava vein mosaic and petunia vein clearing viruses (Shepherd, 1979) also belong to this group.

The virions of most of these viruses have been analyzed and found to contain double-stranded DNA (Shepherd, 1979). Cauliflower mosaic virus has been most fully studied and because of the nature of its genome, which is a double-stranded circular DNA molecule, with a molecular weight of $4.5\text{-}5.0 \times 10^6$ daltons (Meagher, 1977;

Russell *et al.*, 1971; Shepherd, 1971) it could be an ideal vector for plant transformation.

The other group of viruses with DNA genomes is the geminiviruses (Harrison, 1977) and bean golden mosaic virus, is the type member. Other members of this group are beet curly top, cassava latent, Euphorbia mosaic, maize streak, and tobacco leaf curl viruses (Shepherd, 1979). Geminivirus virions contain a single-stranded circular DNA genome that separates into two electrophoretic components and hence may be multi-partite in nature (Shepherd, 1979). The virions are found mainly in the phloem. Beet curly top virus virions (Bennett, 1971) may be restricted to the phloem and cannot be mechanically transmitted between plants.

The DNA genome of geminiviruses is very small, it has a molecular weight of between 6.5 and 9.5 x 10^5 daltons (Goodman, 1977) for golden bean mosaic, 0.7 x 10^6 daltons for maize streak and 0.80 x 10^6 daltons for cassava latent viruses (Harrison, 1977).

All these characteristics make the geminiviruses seem less attractive as potential molecular vectors, but possibly they might be useful.

1.4. SCOPE OF THIS THESIS

In the work described in this thesis I have been concerned with studies that can help the development of systems to be used in plant transformation.

The first step was the selection and detailed study of a possible vector - cauliflower mosaic virus. When my work started, very little was known about the genetic organization of cauliflower mosaic or its biochemistry and a complete knowledge of the virus is essential before it can be used in recombination. In the first four chapters in this thesis I report work done with CaMV, in which I have studied three different strains of the virus and determined and compared the physical and chemical composition of their virions, their infectivity and host range. In order to compare the infectivity of virus preparations it was necessary to develop a reliable assay. To allow reliable comparison between strains and between inoculations, under our conditions, a new local lesion host was sought and found.

The homology of the genome of the three strains was studied by comparing the restriction endonuclease maps for the three strains. The restriction endonucleases selected for the mapping were those that cut the genome in one place so they could be used in cloning the complete genome, and those that could differentiate the three strains. The enzymes used were Eco R1, Hind III, Sal 1, Bam 1, Xho 1, and Xba 1.

While the mapping was in progress, it was observed that if the DNA, in the presence of a very low salt concentration, was heated at 60° for ten minutes and electrophoresed in agarose gels, the molecules would break and give rise to at least three well defined bands. The nature of these bands was investigated and three single-stranded gaps in the DNA were determined and mapped with respect to

the restriction nuclease sites. When the results of Volovitch *et al.* (1978) became available, the methodology was compared and the effect of S_1 nuclease specific for single-stranded DNA was also studied in the 3 strains. The possible biological significance of the single-stranded region was explored by comparing the infectivity of native DNA with cloned DNA in which the gaps have been filled. The effects of cutting and ligation in DNA molecules were studied, and also the possible role of the RNA present in the genome was investigated.

The second part of this thesis, Chapter 5, describes work on the recipient part of a potential transformation system, namely protoplasts, their culture, and the establishment of rapidly growing cell suspension cultures for selection procedures.

Conditions for optimizing DNA absorption was also explored (Chapter 6).

CHAPTER 2.

CAULIFLOWER MOSAIC VIRUS; A Review of Relevant Information.

Cauliflower Mosaic Virus (CaMV) was the first plant virus shown to have a DNA genome (Shepherd *et al.*, 1968).

Taxonomically it is a "caulimovirus" and it is the best known member of this group that contains at least 6 other viruses, whose genomes are double-stranded DNA.

Plant viruses with a DNA genome and CaMV in particular have been reviewed in detail by Shepherd in 1976 and more recently again by Shepherd in 1979, and so in this thesis I will extensively refer to these two reviews.

CaMV has a restricted host range infecting in nature only plants in the family Cruciferae where it gives symptoms from mild mosaic to severe yellowing and deformation of leaves. Two species of Solanaceae have also been experimentally infected. Hill and Campbell (1968) reported that *Nicotiana clevelandii* Gray could be mechanically infected and showed systemic symptoms 2 to 3 weeks after inoculation, however they did not specify which particular strain of CaMV was used and later work (Shepherd, 1979) has shown that only some strains of the virus would infect this species. The other non-crucifer reported to be susceptible is *Datura stramonium* L., which gives necrotic local lesions when inoculated with some strains of the virus (Lung and Pirone, 1972). This reaction provides a quantitative assay for the infectivity of viral isolates, but once again the response is strain specific and very sensitive to environmental conditions (R.D. Brock - personal communication).

CaMV can be experimentally transmitted by sap inoculation but in nature is spread by aphids, of which 27 different species

have been reported to be vectors (Kennedy *et al.*, 1962) suggesting that there is little specificity between the virus and its vector.

Some strains that cannot be transmitted by aphids have been also isolated by Shepherd (1979), but no vector other than aphids have been identified.

Aphid vectors transmit CaMV in a non-persistent manner (Shepherd, 1976); however its transmission characteristics differ in some detail from those of other groups of non-persistent viruses. Insect transmission of CaMV has been studied by several workers (Day and Irsykiewics, 1954; Day and Venables 1961; Hamlyn, 1955; Chalfant and Chapman, 1962) and their studies have been reviewed by Shepherd (1976). Interest in aphid transmission of CaMV has grown since Lung and Pirone (1973; 1974) showed that a (defective) variant of CaMV, which they called CM4-1847, and which could not be transmitted by aphids, was transmissible if the aphids were allowed to feed first on a plant infected with an aphid transmissible strain of CaMV. They suggested that the aphids acquired from the first plant a specific "aquisition factor". They also showed, that aphids probing through an artificial membrane into purified preparations of particles of transmissible strains of CaMV, do not transmit the virus unless first fed on infected plants. This indicated that the virus causes infected plants to produce some vector transmission factor that is not the virus particles themselves (Shepherd, 1979).

CaMV can readily be propagated in several host plants, among others, turnip (*Brassica rapa* L.) and mustard (*Brassica campestris* L.) and the virus can be found in inclusion bodies present in the cytoplasm of infected cells. These refractile inclusion bodies, observed under the light microscope, are easily stained by phloxine (Fujisawa *et al.*, 1967). They may be elliptical, ovoid, lobed or

irregular in shape, and are present in infected cells, being particularly easily seen in epidermal strips of plants, 2-3 weeks after infection. The inclusions are known to be present only in the cytoplasm (Shepherd, 1979) and they seem to contain almost all the viral particles present in the cells. Small amounts of virus particles are free in the cytoplasm and others have been seen in plasmodesmata (Conti *et al.*, 1972). The close association of the virus with inclusion bodies suggests that these are the sites of virus synthesis, or at least virus assembly (Shepherd, 1979). The incorporation of labelled thymine in the inclusion bodies (Kanai *et al.*, 1969) also suggests that the viral DNA replication occurs there.

The inclusion bodies consists of an electron dense granular matrix in which the virions are embedded. The number of virions in each inclusion body varies enormously and is not necessarily associated with the size of the body. The inclusion bodies can be isolated and have been found to consist mainly of a single protein with a molecular weight of about 55,000 daltons (Shepherd, 1979) and this is believed to represent the matrix material of the inclusion. The fact that inclusion bodies of different strains of CaMV have different properties suggest that they are virus-specified and that the matrix protein is a virus-coded product (Shepherd, 1979).

In order to isolate the virus particles, the inclusion bodies must be disrupted. This can be done using detergent and urea during isolation procedures (Hull *et al.*, 1976). However free virus particles tend to aggregate during purification, causing much loss.

The virions of CaMV are isometric and about 50 nm in diameter (Pirone *et al.*, 1961), they have a molecular weight of 22.8×10^6 daltons, their sedimentation coefficient is 208S in 0.1 M NaCl, 0.01 M phosphate,

pH 7.2 at infinite dilution (Hull, Shepherd and Harvey, 1976) but values between 206S to 250S (Shepherd, 1976) have been reported. The buoyant density of the virions is 1.37 gm/ml in caesium chloride and the uncorrected u.v. absorbance at 260 nm radiation is about $7\ mg^{-1}\ ml^{-1}$ (Shepherd, 1970).

CaMV virions are remarkably stable, and are not disassembled by any of the conventional procedures used for this purpose. So far, the genome has only been liberated by proteolysis using pronase or protease in the presence of 0.25% sodium dodecylsulphate or by boiling the particles in 1% sodium dodecylsulphate (Hull and Shepherd, 1977; Shepherd and Wakeman, 1971; Diener and Schneider, 1968; Wilcockson and Hull, 1974; Itoh, Matsui and Hirai, 1969).

Two major proteins, plus small amounts of other polypeptides have been isolated from CaMV virions. The smaller protein has a molecular weight between 32,000 to 42,000 and the larger one 64,000 daltons. Each virion is estimated to have 400 molecules of small protein and 55-60 of the larger protein (Shepherd, 1979). Al Ani *et al.* (1979) suggested that there is only one protein in virions of CaMV - a 42,000 dalton species and that the small polypeptides result from partial proteolysis of the 42,000 species, while the larger proteins are polymers of these. Two minor proteins, MW 56,000 and 49,000 daltons, are also consistently found among the proteins of the virions.

In 1968 Shepherd *et al.* reported the presence of DNA in CaMV virions. Since then, DNA has been shown to be double-stranded, as it has a cooperative-type of melting curve, with a sharp melting point (T_m) of 87.2°C, and a hyperchromicity of 33-36% in SSC. Also it does not react with formaldehyde and has a buoyant density in caesium chloride of 1.702 g/ml (Shepherd *et al.* 1970), which suggests that

it has a G-C content of about 43% (Schildkraut *et al.*, 1962).

The molecular weight of the DNA as deduced from sedimentation studies and electron microscope measurement, is between 3.6 and 5×10^6 daltons (Shepherd and Wakeman, 1971; Russell *et al.*, 1971; Hull and Shepherd, 1977).

The DNA extracted from virions is a mixture of circular and linear molecules. The circular molecules show different degrees of "twisting" (not supercoiling) but all have a mean length reported by Shepherd and Wakeman (1971) to be 2.31 µm and by Russell *et al.* (1971) to be 2.47 µm. However when analysed by gel electrophoresis in polyacrylamide and agarose gels they seem heterogenous and separate into two major components (Volovitch *et al.*, 1978; Civerolo and Lawson, 1978). Furthermore, solutions of CaMV DNA sediment as two components with sedimentation coefficients of 17.1S and 19.0S. Infectivity is associated with the slower sedimenting component (Hull and Shepherd, 1977) and this is the circular form observed in electron micrographs.

The heterogeneity in electrophoretic mobility has not been correlated with any particular structural feature, but is probably caused by local conformational differences (Volovitch *et al.*, 1978).

The CaMV DNA contains a small amount (less than 1%) of covalently linked RNA (Hull and Shepherd, 1977) whose position in the genome is unknown.

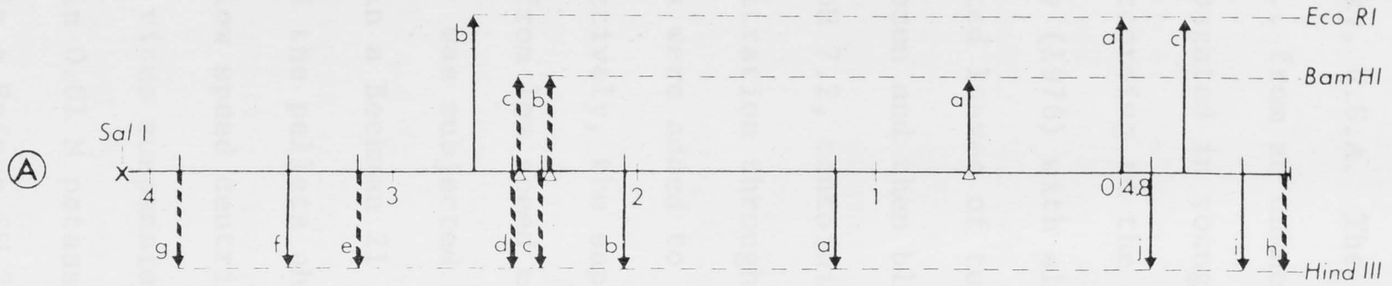
Volovitch *et al.* (1978) showed that there are three single-stranded gaps in the genome of CaMV. When the DNA was electrophoresed under alkaline conditions no circular molecules were found but there were three linear fragments whose combined length was that of the intact genome. This indicated that neither DNA strand is covalently closed,

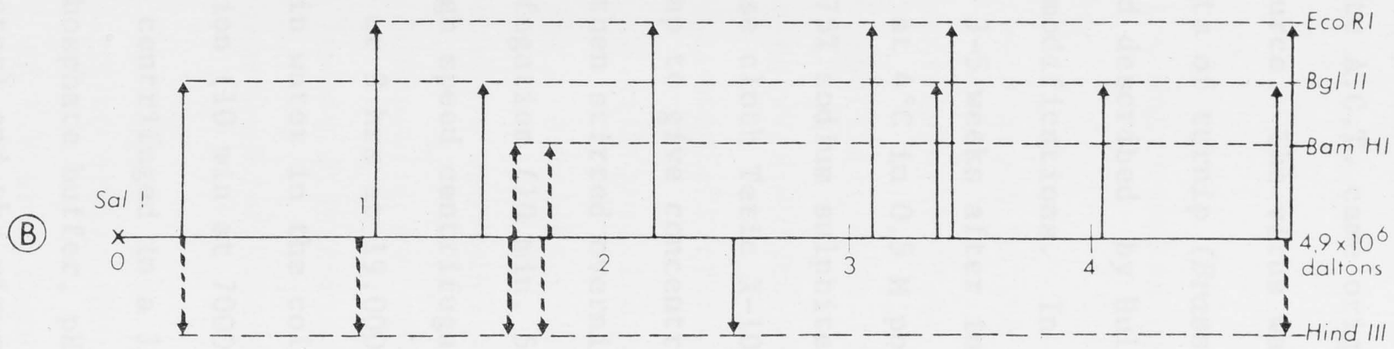
and that there are three discontinuities in the molecules. These gaps are susceptible to S_1 nuclease and are not randomly located. The biological significance of these gaps is still unknown.

Electrophoretic analysis of CaMV DNA treated with various specific restriction endonucleases has enabled maps of the genome to be deduced (Meagher *et al.*, 1977; Hull and Howell, 1978; Volovitch *et al.*, 1978; Lebeurier *et al.*, 1978). These studies have been carried out primarily on the strain Cabbage B and Lebeurier *et al.* (1978) summarized the main differences between variants of this strain on the graph reproduced in Fig. 1.

Different strains or variants within one strain have different restriction sites for some enzymes and a particular part of the genome seems to have greater frequency of sequence variation than the rest of the molecule. Heterogeneity within strains has been repeatedly reported with just part of the DNA population having enzyme sensitive sites (Meagher *et al.*, 1977a). This has been further studied by examination of fragments of CaMV DNA cloned in *E. coli* (Meagher *et al.*, 1977b) and it has been suggested that base substitution or modification, inversions or small deletions may account for the variations in sequences.

Fig. 1. Fig 7 from "Physical map of DNA from a new Cauliflower Mosaic Virus Strain" (Lebeurier *et al.*, 1978). This diagram compares the restriction endonuclease map of their Cabb-S isolate of CaMV and that described by Meagher *et al.* (1977a).





Comparison between the cleavage maps of the DNA of two CaMV isolates. (A) Cabb-B, CaMV strain described by Meagher et al. (1977). (B) our Cabb-S, CaMV strain. We have linearized the two genomes starting at the *Sal*I site in order to simplify the comparison. Broken arrows refer to sites of cleavage common to both maps.

CHAPTER 3

MATERIALS AND METHODS

3.1. ISOLATION AND STORAGE OF THE VIRIONS OF THE VIRUS

The isolates New York 8135 and Campbell were obtained from Dr. R.D. Brock, CSIRO, Canberra, and originally came from Dr. Pirone, Kentucky, U.S.A. The isolate A.C.T. came originally from Scotland, U.K., from an unknown source. The virus was maintained and propagated in young plants of turnip (*Brassica rapa* cv. Just Right) according to the method described by Hull, Shepherd and Harvey (1976) with slight modifications. In essence the method was: infected leaves of turnip, 3-5 weeks after inoculation were chilled or frozen and then blended at 4°C in 0.5 M potassium phosphate buffer, pH 7.2, containing 0.75% sodium sulphite (1 ml/g leaf tissue). After filtration through cheese cloth Teric X-10 (ICI Australia) and urea were added to the sap to give concentrations of 2.5% and 1 M respectively, the sap was then stirred overnight. The supernatant fluid from low speed centrifugation (10 min, 5000 rpm in a Sorval GSA rotor) was subjected to high speed centrifugation (2.5 hrs at 20,000 rpm in a Beckman 21 rotor or 3 hrs at 19,000 rpm in the same rotor) and the pellets shaken in water in the cold until suspended. After low speed centrifugation (10 min at 7000 rpm in a Beckman rotor) the virus suspension was centrifuged in a 10 to 40% sucrose gradient (in 0.01 M potassium phosphate buffer, pH 7.2; 2.5 hrs at 23,000 rpm in a Spinco SW 25.1 rotor) and the virus-containing band collected using a Varian Techtron UV-VIS model 635 spectrophotometer, with a Zeiss 80 µl volume flow-through cell. The virus in sucrose was diluted 1:1 with water and pelleted by high speed centrifugation (1 hr at

47,000 rpm in a Beckman Ty 65 or Beckman Ti 50 rotor). The pellets were resuspended in water. When the virions were purified from fresh leaves centrifugation in a second sucrose gradient was necessary to obtain virions free of plant material.

The purified virions, resuspended in water, were stored either frozen at -20°C or at 4°C after adding a crystal of thymol phenol blue or a drop of 1% sodium azide solution.

3.2. INFECTIVITY ASSAYS

The infectivity of the viral isolates was measured by a local lesion assay developed for our conditions.

Seedlings of turnip cv. Just Right were grown in the glasshouse at a temperature between 15-25°C, with only natural light and were inoculated ten days after planting, when the first true leaves were approximately 4-5 cm long. The leaves were dusted with 400 mesh Carborundum before being inoculated, using a sterile cotton bud, dipped in sap from infected plants or with purified virion preparations at a concentration of 1 mg/ml. For infectivity comparisons, the sap or the virion preparation was used, together with a series of 10 fold dilutions; each inoculum was applied on four or more half leaves or eight whole leaves distributed between plants to minimize interleaf and interplant susceptibility differences.

The sap extract and the inoculum dilution series were made using 0.01 M potassium phosphate buffer, pH 7.2 as diluent.

After inoculation, the plants were shaded by covering them with two thicknesses of brown wrapping paper for a least 19 hrs. The lesions were counted 8 days after inoculation.

3.3. HOST RANGE

The three strains were inoculated on turnip (*Brassica rapa* cv. Just Right), rapeseed (*Brassica napus* cv. Masowiecki, Zephyr, Turret, Target), mustard (*Brassica perviridis* cv. Tendergreen), different races of *Arabidopsis thaliana* including Kologne, Nantes, Eastland, Columbia, Innsbruck, *Nicotiana clevelandii* (4 isolates available from the Australian Collection of Nicotiana and a selection from the Waite Institute, Adelaide, a gift from Dr. R.I.B. Francki), *Nicotiana bigelovii*, *N. glutinosa*, *N. debneyii* and commercial tobacco (*N. tabacum* cv. Hicks).

3.4. SEDIMENTATION COEFFICIENTS

The sedimentation coefficients of purified virions were determined in an analytical centrifuge Spinco Model E in water, at 20°C. The cells were scanned photometrically every two minutes when centrifuging at 30,000 rpm or every 3 min. when at 20,000 rpm.

3.5. NUCLEIC ACID METHODS

3.5.1. *Preparation of CaMV genome DNA*

DNA was purified from virions using the method outlined by Shepherd , Bruening and Wakeman (1970). In essence, the viral suspensions (1-2 mg/ml) in 1 x SSC were incubated with 500 µg of Pronase (predigested at 37°C for 2 hrs). Then enough sodium dodecyl sulphate (SDS) was added to the solution to give a concentration of 0.5%. After incubating the preparation overnight at room temperature this was placed in an ice-water bath for 30-40 min in order to precipitate some of the SDS. This was removed by centrifuging at 5000 rpm for 5 min and the suspension extracted twice with phenol. To the aqueous phase containing the DNA was added 1/10 volume of 3 M

sodium acetate pH 5.5 and two volume of absolute ethanol. The mixture was kept at -20°C for at least 2 hrs or at -70°C in a dried ice-ethanol bath for 20 min, then centrifuged for 30 min at 10,000 rpm in a Sorval HB4 rotor and the precipitated DNA resuspended in 10 mM Tris-1 mM EDTA buffer (TE buffer), pH 8.4, dialyzed against many volumes of the same buffer and stored in acid-washed glass tubes at 4°C. The concentration of DNA preparations was calculated from their optical absorbance at 260 nm (1 µg DNA = 0.02 O.D.) and their purity estimated by the A_{260}/A_{280} ratio (DNA = A_{260}/A_{280} = 2), by its homogeneity when electrophoresed and occasionally by observation under the electron microscope.

3.5.2. *Digestion of DNA by specific nucleases*

The restriction endonucleases Hind III, Bam I, Sal I, Xho I, Xba I were obtained from Biolab and Eco R1 from Boehringer-Mannheim.

DNA fragments of the isolate Campbell were mapped first and later the other two strains compared to it.

Each digestion mixture (40 µl) contained 2-3 units of each enzyme, 1-2 µg of DNA and was incubated at 37°C for 1 hr for Eco R1, Hind III, Sal I, but for Bam I, Xho I and Xba I the mixtures were incubated for 45 min, then 2-3 more units of enzyme was added and the digestion continued for another 45 min. The compositions of particular reaction mixtures used for each enzyme are summarized in Table 3.1.

Digestion was stopped by heating the sample at 60°C for 5 min then adding 10 µl of a freshly prepared mixture of 0.1% bromophenol blue, 50% glycerol and 0.5 M EDTA.

TABLE 3.1. Reaction mix used in digesting CaMV DNA with different restriction endonucleases.

Enzyme	Bacterial source *	Cleavage sequence* 5' 3'	Buffer Tris-HCL	pH	$MgCl_2$	NaCl	β mercapto-ethanol	Bovine serum albumin µg/ml
Eco Rl	*Escherichia coli* RY 13	G↓AATTC	12.5 mM	7.5	12.5 mM	50 mM	-	-
Hind III	*Haemophilus influenzae* Rd.	A↓AGCTT	12.5 mM	7.5	12.5 mM	100 mM	10 mM	50
Sal I	*Streptomyces albus*	G↓TCGAC	10.0 mM	7.0	10.0 mM	100 mM	10 mM	-
Bam I	*Bacillus amyloliquifaciens* H.	G↓GATCC	6.0 mM	7.4	6.0 mM	50 mM	6 mM	100
Xba I	*Xanthomonas bachii*	T↓CTAGA	6.0 mM	7.9	6.0 mM	150 mM	6 mM	-
Xho I	*Xanthomonas holicola*	C↓TCGAG	6.0 mM	7.4	6.0 mM	150 mM	6 mM	100

* Roberts (1976); arrow indicates cleavage point.

S_1 nuclease was treated differently. DNA (1-2 µg) and S_1 nuclease were incubated in 30 µl of 0.03 M sodium acetate, 0.05 M NaCl and 3 mM $ZnSO_4$, pH 4.6, for 60 min. The reaction was stopped by the addition of 5 µl of 0.25 M EDTA, pH 8.0.

3.5.3. *Analytical gel electrophoresis*

Neutral gels: DNA fragments were electrophoresed into cylindrical gels (11.5 x 0.7 cm) containing 0.8% agarose (Sigma) in 40 mM Tris acetate pH 8.4, 20 mM sodium acetate, 2 mM EDTA and 1 mg/l of ethidium bromide. Electrophoresis was done at room temperature and the buffer tanks contained a total of 2 l of the above buffer to give stability and improve resolution. A current of 5 mA/gel (50-70 volts) was applied for 3-4 hrs. Gels were examined and photographed under UV light using Polaroid Type 665 positive/ negative black and white film, and Kodak red and yellow filters (#23 and 15, respectively).

Alternatively, polyacrylamide was used as the supporting material for some neutral gels. The acrylamide was polymerized to form horizontal slab gels of 6% gel or vertical slabs of 10% (w/v) polyacrylamide. Both were electrophoresed overnight using 12 mA-30 volts and the same buffer as the agarose gels but without ethidium bromide. The gels were stained afterwards for 20 min in a solution of the same buffer plus 1 mg/l of ethidium bromide.

Densitograms of the Polaroid negatives were obtained using a Joyce-Loebl microdensitometer and the areas under peaks compared.

The molecular weights of the restriction fragments were determined by comparing their rates of migration with co-electrophoresed standards. These were *E. coli* λ DNA digested with both Hind III

and Eco R1 (Murray and Murray, 1975) and/or Col E_1 digested with Hae III (Eshaghpour and Crothers, 1978) (Fig. 2).

Alkaline gels: Cylindrical gels were used containing 0.8% agarose in 30 mM NaCl, 2 mM EDTA (McDonell, Simon and Studier, 1977) and as running buffer 20 mM NaOH-2 mM EDTA was used. Electrophoresis was done and assessed using the same conditions as those used for the neutral gels except that after each run the gels were stained for 20 min with ethidium bromide (1 mg/l) dissolved in the running buffer and then photographed.

Different standards were used in alkaline gels; these were phage λ genome DNA digested by Eco R1, or with both Eco R1 and Hind III, also ϕX 174 digested with Hae III or Pst I (Godson 1976, Hayashu *et al.*, 1974).

3.5.4. *Recovery of DNA fragments from agarose gels*

The DNA fragments were separated by electrophoresis in gels made from 0.8% low melting point agarose (Sea Plaque MCI) using the same neutral buffer and conditions described before, but with only 0.025% ethidium bromide.

The position of the DNA bands was determined by their fluorescence under UV light and the bands were cut from the gels and frozen. Then the DNA was recovered from the gels by one of three methods:

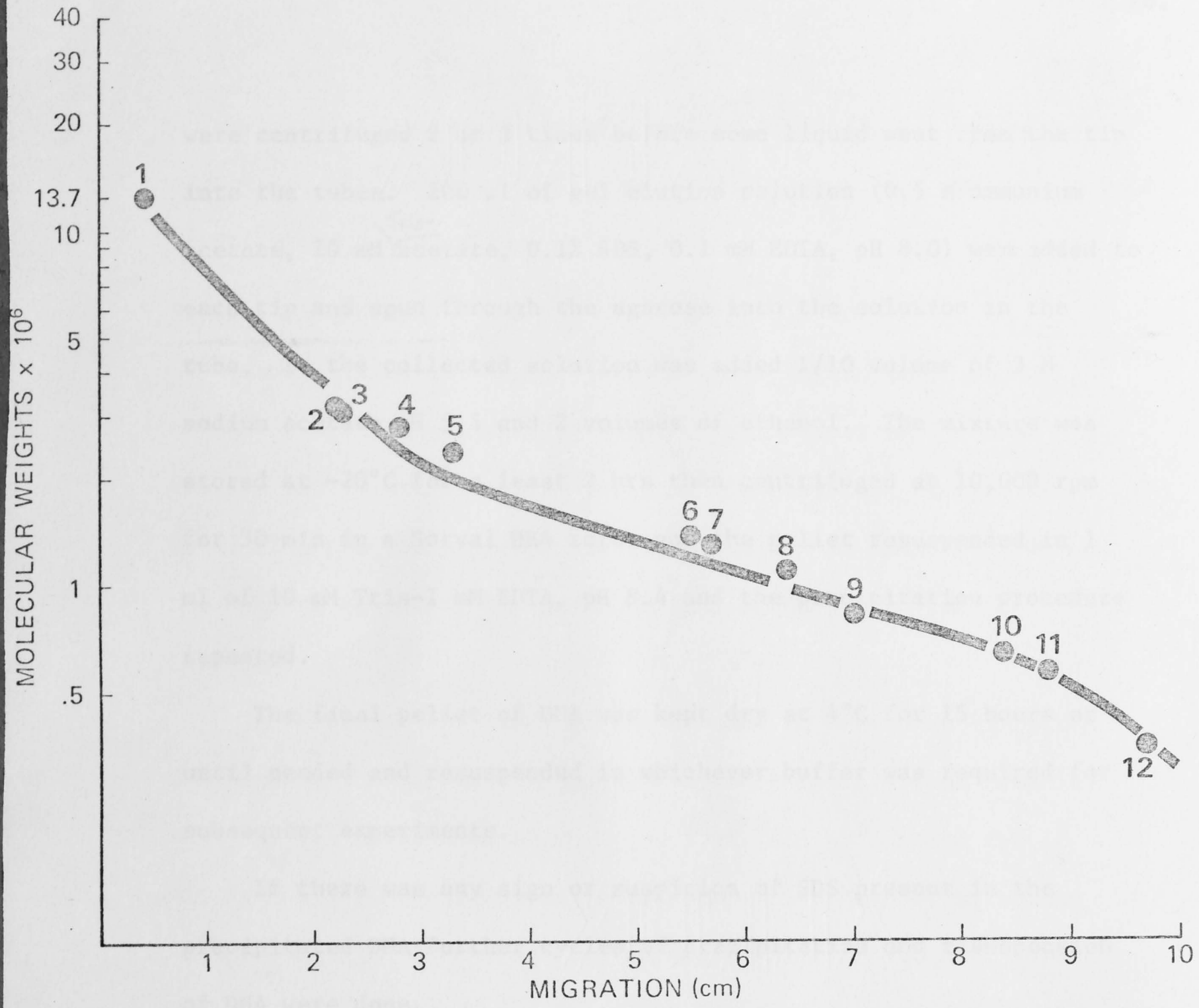
(a) Freeze-squeeze method: (E. Dennis - personal communication)

Frozen agarose blocks were placed in plastic disposable pipette tips previously plugged with siliconized glass wool, and left to thaw at room temperature for about 30 min. After breaking up the agarose with a glass rod, the tips were placed in siliconized glass tubes and spun at 3000 rpm for 5 min. Sometimes the tips

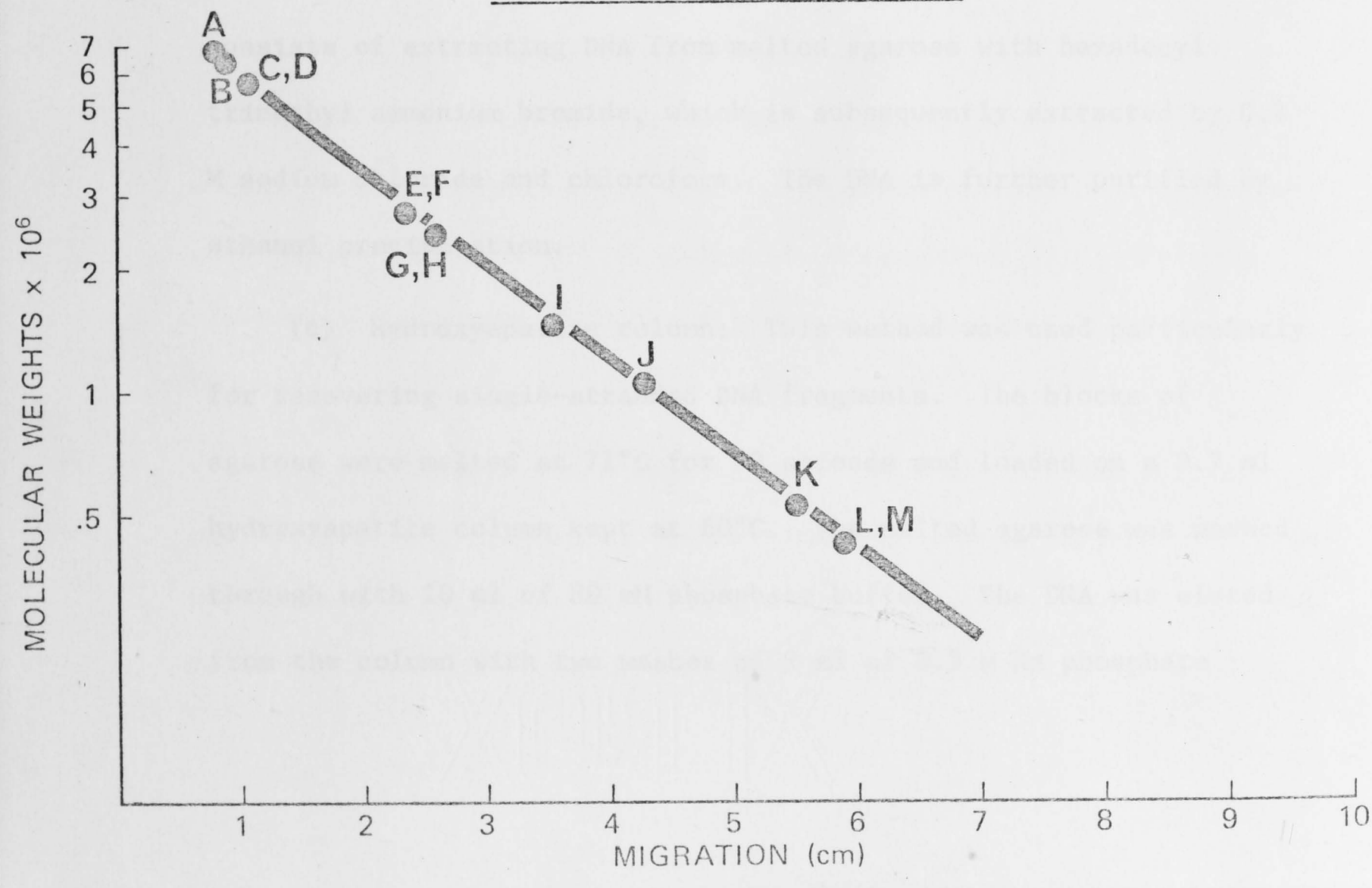
Fig. 2. Graph showing relationship between molecular weight of the restriction endonuclease fragments and their electrophoretic mobility in agarose gels; the distance migrated by the DNA fragments is plotted against the logarithm of their molecular weights. The molecular weight of CaMV DNA fragments were estimated by comparison with those of λ phage DNA double digested with Eco Rl and Hind III (upper figure) or with Col El DNA digested with Hae III (lower figure). The molecular weight of the λ fragments after digestion were (x 10^6 daltons): 1 = 13.7; 2 = 3.19; 3 = 3.07; 4 = 2.79; 5 = 2.41; 6 = 1.34; 7 = 1.29; 8 = 1.07; 9 = 0.91; 10 = 0.63; 11 = 0.56; 12 = 0.44 (Murray and Murray, 1975).

The molecular weights of the Col E_1 fragments after digestion with Hae III (x 10^6 daltons) were: A = 0.68; B = 0.64; C,D = 0.56; E,F = 0.272; G,H = 0.247; I = 0.154; J = 0.106; K = 0.052; L,M = 0.043; N = 0.026 (Eshaghpour and Crothers, 1978).

λ DNA DOUBLE DIGESTED WITH $EcoR_1$ AND HIND III



Col E_1 DIGESTED WITH Hae III



were centrifuged 2 or 3 times before some liquid went from the tip into the tubes. 200 µl of gel elution solution (0.5 M ammonium acetate, 10 mM Tris-acetate, 0.1% SDS, 0.1 mM EDTA, pH 8.0) were added to each tip and spun through the agarose into the solution in the tube. To the collected solution was added 1/10 volume of 3 M sodium acetate pH 5.5 and 2 volumes of ethanol. The mixture was stored at -20°C for a least 2 hrs then centrifuged at 10,000 rpm for 30 min in a Sorval HB4 rotor and the pellet resuspended in 1 ml of 10 mM Tris-1 mM EDTA, pH 8.4 and the precipitation procedure repeated.

The final pellet of DNA was kept dry at 4°C for 15 hours or until needed and resuspended in whichever buffer was required for subsequent experiments.

If there was any sign or suspicion of SDS present in the precipitated DNA further cycles of precipitation and resuspension of DNA were done.

(b) Langridge method: This method (Langridge *et al.*, in press) consists of extracting DNA from melted agarose with hexadecyl trimethyl ammonium bromide, which is subsequently extracted by 0.2 M sodium chloride and chloroform. The DNA is further purified by ethanol precipitation.

(c) Hydroxyapatite column: This method was used particularly for recovering single-stranded DNA fragments. The blocks of agarose were melted at 71°C for 90 seconds and loaded on a 0.7 ml hydroxyapatite column kept at 60°C. The melted agarose was washed through with 10 ml of 80 mM phosphate buffer. The DNA was eluted from the column with two washes of 2 ml of 0.5 M Na phosphate

buffer, and dialyzed extensively against TE buffer to remove the phosphate, precipitated with ethanol in the usual way and resuspended in the appropriate buffer.

3.5.5. *DNA melting*

To determine the temperature at which intact CaMV genome melted, the DNA was partially denaturated by heating the DNA samples for 10 min at a wide range of temperatures, between 40-98°C. The DNA was suspended in 1 mM Tris 0.1 mM EDTA, pH 8.4 and the samples cooled by placing them in ice and loading in the gels immediately.

To determine the sizes of DNA strands in molecules of intact CaMV genome and in DNA fragments, they were totally denatured in 1 mM Tris, 0.1 mM EDTA or 10 mM Tris, 1 mM EDTA by heating for 10 min in boiling water, quickly cooled in an ice bath or by adding 1/10 volume of 1 M NaOH and incubating at 37°C for 10 min. Both methods produced denatured DNA which was then layered onto neutral or alkaline gels for fractionation.

3.5.6. *Reannealing of intact CaMV genome DNA*

To DNA melted by heating was added enough SSC or NaCl to give a final concentration of 0.2 M salt. The mixture was then incubated at 60°C for 30 min to 2 hrs, followed by 30 min at 37°C, glycerol, bromophenol blue were added as before and the samples layered on the gels.

Single-stranded DNA fragments were isolated from low melting agarose gels and treated in the way just mentioned. Alternatively the appropriate slices were cut from the gel, melted together at 71°C for 90 seconds, the salt concentration taken to 0.2 M, with SSC or NaCl,

and after incubation as before, loaded quickly onto gels with warmed micropipettes.

3.5.7. *Electron microscopy of isolated DNA fragments*

The fragments obtained after heating the CaMV DNA were prepared for electron microscopy by binding them to a protein film as described by Davis *et al.* (1971). Each sample was spread under aqueous conditions, or in 50% formamide.

3.5.7.1. Spreading under aqueous conditions

0.5 µg/ml of DNA was mixed with 0.5 M ammonium acetate, 1 mM EDTA, pH 7.4 and 0.1 mg/ml of cytochrome C. Ten µl of this mix were spread on 0.25 M ammonium acetate.

3.5.7.2. Formamide spreading

The DNA was mixed with 0.1 mg/ml of cytochrome C, 0.1 M Tris-10 mM EDTA, pH 8.4 in 50% formamide. Ten µl of this solution were spread on 20% formamide in 10 mM Tris-1 mM EDTA, pH 8.4.

In both cases the protein film was collected on a coated copper grid (400 mesh) and stained in 5×10^{-5} M uranyl acetate in 90% ethanol for 30 seconds and rinsed for 10 seconds in isopentane. The grids were rotationally shadowed with platinum paladium at low angle and examined in 1-L Phillips EM 200 electron microscope.

The length of the fragments was calculated from traces of a 35 mm film projected on a screen, measured with a map measurer. The magnification was estimated in comparison with a graticule (2160 lines/mm).

3.5.8. *Hybridization of Eco R1 fragments and single stranded fragments from denatured DNA to total digests of CaMV DNA with Eco R1 and completely denatured DNA*

3.5.8.1. Preparation of [^{32}P]-cRNA

[α-^{32}P] labelled ribonucleoside 5'-triphosphates were prepared as described by Symons (1977).

The cRNA copies to the different fragments were synthesised from approximately 1.2 µg of DNA of each fragment recovered from 10 gels (R. Appels - personal communication).

Fifteen nmoles each of α-[^{32}P] ATP, α-[^{32}P] CTP, α-[^{32}P] UTP, and GTP were added to the DNA and the suspension dried in a rotary evaporator. The dried mix was resuspended in 100 µl of 40 mM Tris, pH 7.9, 5 mM $MgCl_2$, 80 mM KCl, 1 mM DTT and after adding *E. coli* RNA polymerase was incubated for 1-2 hrs at 37°C. The incorporation of radioactivity was checked at 30 min intervals by comparing the total radioactivity in the sample with the radioactivity incorporated in filters after acid precipitating 1 µl aliquots of the preparation. When incorporation of labelled ^{32}P ceased, the sample was treated with DNAse and after 30 min the RNA was deproteinized by a phenol extraction and passed through a Sephadex G75 column. The labelled RNA after elution was mixed with 0.25 M NaCl and 0.3 M Na acetate and two volumes of absolute ethanol, kept at -20°C for 2 hrs, centrifuged to sediment the precipitate, which was then resuspended in 6 x SSC, and kept frozen at -20°C until used.

3.5.8.2. Hybridization of labelled cRNA

CaMV DNA digested with Eco R1 and DNA denatured by heating or alkali treatment was electrophoresed in horizontal slab gels

(0.8% agarose) and transferred to nitrocellulose strips by the Southern technique (Southern, 1975), dried and hybridized with the appropriate ^{32}P cRNA. The hybridization mixture contained 3 x SSC, 50% formamide and 0.1% SDS and was incubated at 45°C overnight. After removing the unbound cRNA with pancreatic RNAse, the filters were exhaustively washed in 2 x SSC, 0.1% SDS, dried, and placed against Kodak RP Royal X-ray film, for autoradiography.

3.5.8.3. Determination of the T_m of the RNA-DNA hybrids

Knowledge of T_m of the hybrids was required so that very stringent conditions for the hybridization procedures could be used.

^{32}P cRNA from Eco R1 fragment 2.1 and 1.3 was hybridised in 3 x SSC, 50% formamide, 0.1% SDS at 45°C for 3 hrs to total CaMV DNA fixed to nitrocellulose filters (Birnstiel *et al.*, 1972).

The T_m of the RNA-DNA hybrids was determined by transferring the filters, at 10 min intervals, into 0.7 ml aliquots of 3 x SSC, 50% formamide at increasing temperatures. The released radioactivity was measured in a scintillation counter after adding 0.3 ml of H_2O and 9.0 ml of Triton X-100 based scintillation fluid (Appels *et al.*, 1978).

3.5.9. *"Cutting" and "ligation" of the CaMV genome DNA.*

The DNA was cleaved with the selected restriction endonuclease under appropriate conditions (See Section 3.5.3. in this Chapter). After incubation the volume of the reaction was brought to 1 ml with glass distilled water and 100 µl of 0.67 M Tris-0.067 M $MgCl_2.6H_2O$ was added. The sample was heated at 65°C for 5 min and slowly cooled to 4°C. When at 4°C, dithiothreitol (0.67 mM), ATP (0.067 mM), bovine serum albumin (1 µl of 1 mg/ml solution) and 2 µl

of T_4 ligase (in glycerol) were added and the solution ligated by incubation at repeated cyclic temperatures which run from 4° to 12°C overnight. The effectiveness of ligation was checked by comparing electrophoresed DNA before cutting, after cutting and after ligation.

3.5.10. *"Filling" of single-stranded gaps in CaMV DNA using DNA polymerase*

The procedure followed was based on the method used to obtain radioactive labelled DNA copies *in vitro* by nick translation (Rigby *et al.*, 1977; E. Dennis, personal communication).

DNA (1-3 µg) was mixed with 7.5 nmoles d ATP, 7.5 nmoles d CTP, 7.5 nmoles d GTP, 7.5 nmoles d TTP. After drying the sample in a rotary evaporator and resuspending in water twice it was finally resuspended in 100 µl of 50 mM sodium phosphate, 7 mM $MgCl_2$ and 5 µl of DNA polymerase I (a gift of Dr. D.L. Brutlag, Stanford University) were added. The solution was incubated at 14°C for 30 min and 3 hrs.

3.5.11. *Effect of ribonuclease H on CaMV DNA*

The reaction mixtures (50 µl) of 36 µg DNA contained 1 µM of Tris-HCl (pH 8.0), 0.5 µM of $MgCl_2$, 0.3 µM of dithiothreitol, 2 µg of bovine serum albumin and 0.5 units* of RNase H and were incubated for 1 hr at 37°C (Leis *et al.*, 1973). Then, 1 µg of DNA was directly electrophoresed in a neutral gel, and 1 µg was denatured by adding $^1/_{10}$ volume of 1 M NaOH before electrophoresis in neutral gels. The rest of the treated DNA was inoculated to turnip seedlings for infectivity assay.

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* 1 unit of RNase H produced 10^{-9} mol of nucleotide in 20 min. at 37°C.

3.5.12. *Preparation of pancreatic RNase A solutions free of DNases*

A stock solution (2 mg/ml) of Sigma Pancreatic RNase A was prepared in 0.1 M NaCl, 0.01 M Na acetate, pH 5.1. The solution was then heated to 80°C for 10 min in a stoppered tube, cooled and stored frozen at -20°C.

3.5.13. *Effect of pancreatic ribonuclease A and T_1 on CaMV DNA*

30-40 µg of DNA was incubated in the same buffer as for ribonuclease H with 2 or 4 µg of RNase A and with 200 units of ribonuclease T_1 (Boehringer-Mannheim). The preparations were incubated at 37°C for different lengths of time according to the treatment and then used to inoculate turnip seedlings for infectivity assay. As for ribonuclease H, 1 µg of DNA was electrophoresed in neutral gels and another µg denatured prior to electrophoresis.

3.5.14. *Infectivity assay for DNA*

Turnip seedlings (cv. Just Right), mustard, (cv. Tendergreen) and rapeseed (cv. Masowiecki) were inoculated with DNA solutions using sterile cotton swabs dipped in the inoculum. As an abrasive, sterile Celite was added to each solution to a concentration of 10 mg/ml. Different amounts of DNA (10-40 µg) were mixed with sterile 1 x SSC to give a volume of 0.5 ml, enough to inoculate 32 seedlings.

The conditions for inoculation were the same as those used in local lesion assays. The plants were examined for symptoms at weekly intervals up to 5 weeks after inoculation.

CHAPTER 4.

4.1. PROPERTIES OF THE VIRIONS OF CAULIFLOWER MOSAIC VIRUS

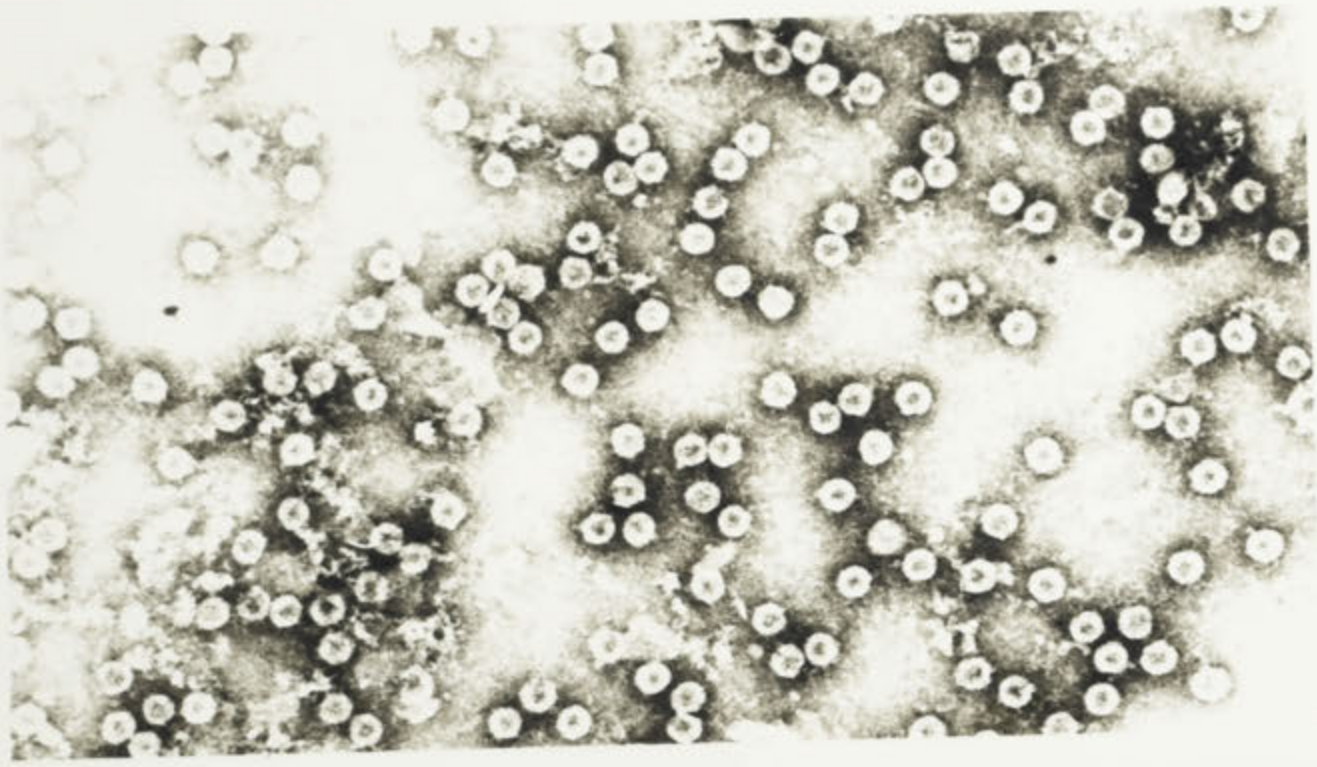
4.1.1. *Virion purification and storage*

The yield of virions of the three isolates was variable. The best yields were obtained from New York 8135 (10-12 mg of virions/kg of infected fresh leaf tissue). If frozen leaves were used for isolation the yield was inversely proportional to the length of storage, at least up to a period of 4 months. The yield of the Campbell strain was less than that of New York, 6-10 mg of virions/kg of fresh leaves, and this was very much reduced if the plant material had been frozen. The isolate A.C.T. was the most difficult to handle experimentally, yielding only 3-5 mg of virions/kg of fresh infected leaves and with complete loss of the virions if the leaves were frozen.

The purified virions appeared as light-scattering bands in the middle of the 10-40% sucrose gradient, after centrifuging for 2.5 hrs at 23,000 rpm. This band was well separated from the plant material, especially when the preparations were made with leaves that had been frozen before extraction, but since freezing depressed the yields this procedure was abandoned. However if the virions after gradient purification were still contaminated with any green plant material the preparation was further purified by centrifuging it through another 10-40% sucrose gradient. The purity of the preparation was assessed by measuring its light absorbance at A_{260} and A_{280}. The A_{260}/A_{280} ratio of the most pure preparations was 1.40 - 1.43 which conforms with the values presented by Shepherd (1970). Occasionally, the preparations were checked in an electron microscope after negative staining (Fig. 3).

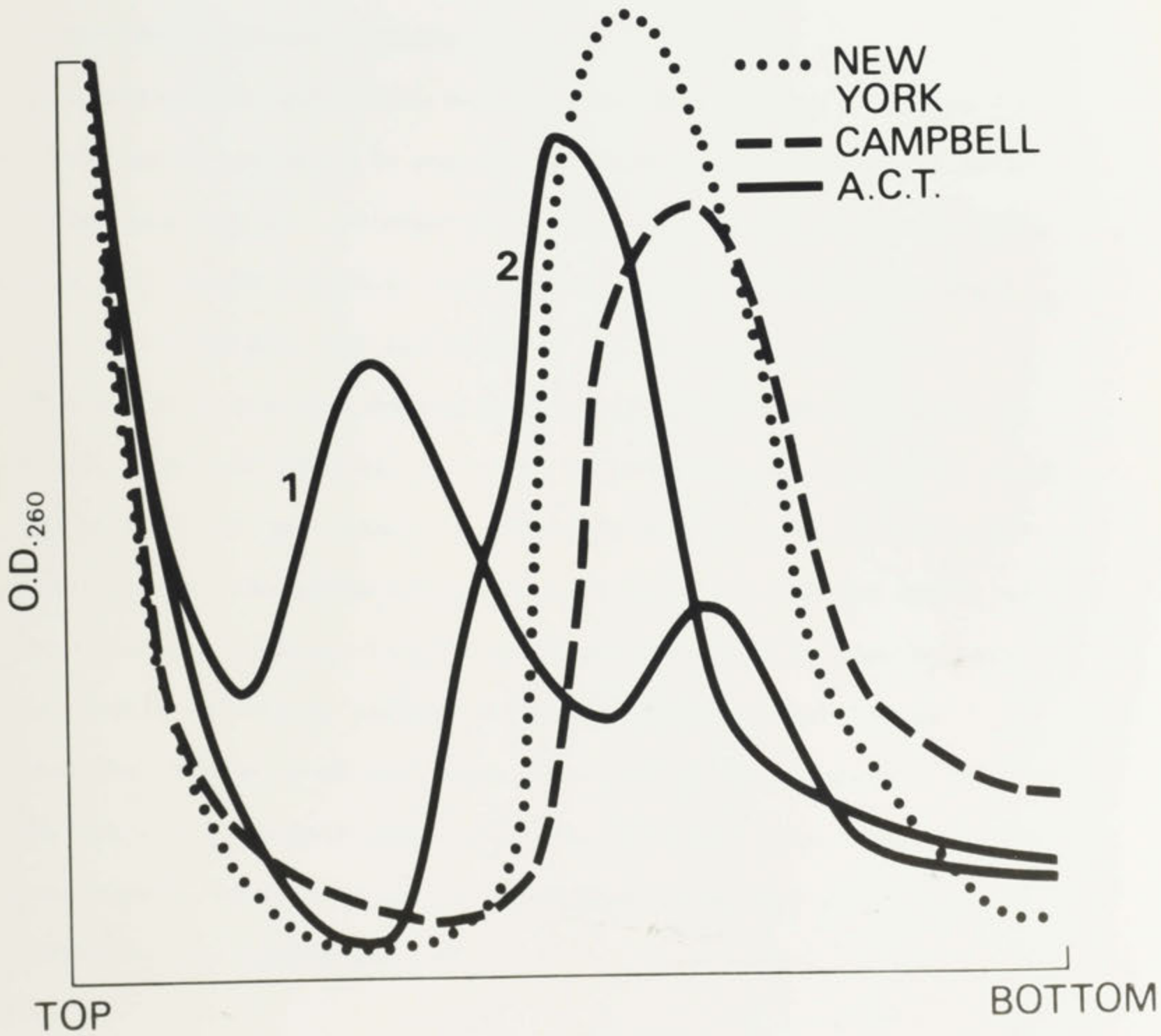
Fig. 3. Electron micrograph of CaMV virions negatively stained with 1% phosphotungstic acid, pH 7.1.

Fig 4. A graph showing the absorbance of typical sucrose gradient purifications of New York, Campbell and A.C.T. virions. Graph 2 for A.C.T. is the more common profile. In some experiments where the infected plants were older a second slower migrating peak (1) appeared. Centrifugation on 10-40% sucrose gradients was for 2.5 hrs. at 23,000 rpm at 0°C in a Beckman SW 25.1 rotor.



CaMV
Particle size = 50nm

1μ



The sucrose gradient profiles for typical samples of the isolates New York 8135 and Campbell are shown in Fig. 4, they show that the virions sedimented to give an asymmetrical absorption peak. The degree of asymmetry depended on the age and condition of the plants from which the virions were isolated. The asymmetry of the peak for the A.C.T. isolate was more conspicuous and in some experiments a second slower migrating peak was present (Fig. 4).

To check the possibility that the second peak was a contaminant virus, the particles from each peak were examined under the electron microscope and found to be indistinguishable in diameter and appearance. The infectivities of samples of the two peaks were similar and gave plants with indistinguishable symptoms.

The nucleic acid which was isolated from virions from each peak proved to be resistant to pancreatic RNAse A and sensitive to DNAse I when examined by electrophoresis (Fig. 5). The DNA isolated from each peak had an identical restriction endonuclease pattern, which was coincident with the map prepared for the isolate (Fig. 6). This evidence strongly indicates that both peaks contained only the A.C.T. strain of CaMV and there was no contamination by another virus. The only factor that seemed to have some influence on the sedimentation rate of the particles was the age of the plants and degree of infection. Old plants (5 to 6 weeks after infection) showing very noticeable chlorosis, yielded virion preparations that had an increased asymmetry of the virus peaks in sucrose gradients and for the A.C.T. isolate gave two peaks. However, this phenomenon was not consistent. Other host and/or environmental factors probably also influence this phenomenon.

Fig. 5. Agarose gels (0.8%) into which has been electrophoresed A.C.T. genomic DNA treated with DNase I nuclease and pancreatic ribonuclease A.

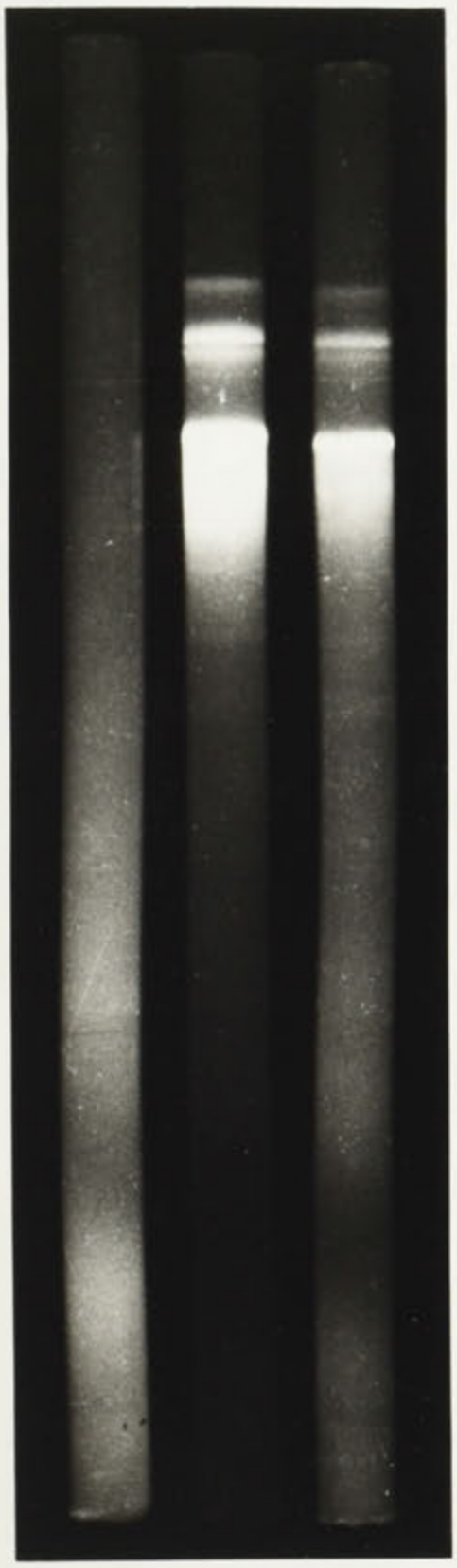
(a) DNA treated with DNase I

(b) DNA isolated from the slower migrating peak, (Graph 1, Fig. 4) and then treated with RNase A.

(c) DNA isolated from the faster migrating peak, (Graph 2, Fig. 4) and then treated with RNase A.

Fig. 6. Polyacrylamide gels (10%) (a,b) and agarose gels (0.8%) (c,d) of A.C.T. DNA isolated from the two peaks (seen in Fig. 4) with different sedimentation coefficients and digested with restriction endonuclease Hind III (a,b) and Eco R1 (c,d). No differences were detected between the two DNAs. In tracks c and d the Eco R1 fragment of A.C.T. are as indicated. The remaining bands are Eco R1 digests of λ used as an internal standard (see Materials and Methods).

Fig 5


◀ TWISTED MOLECULES
◀ CIRCULAR MOLECULES
◀ LINEAR MOLECULES
4.8 x 10⁶
a b c

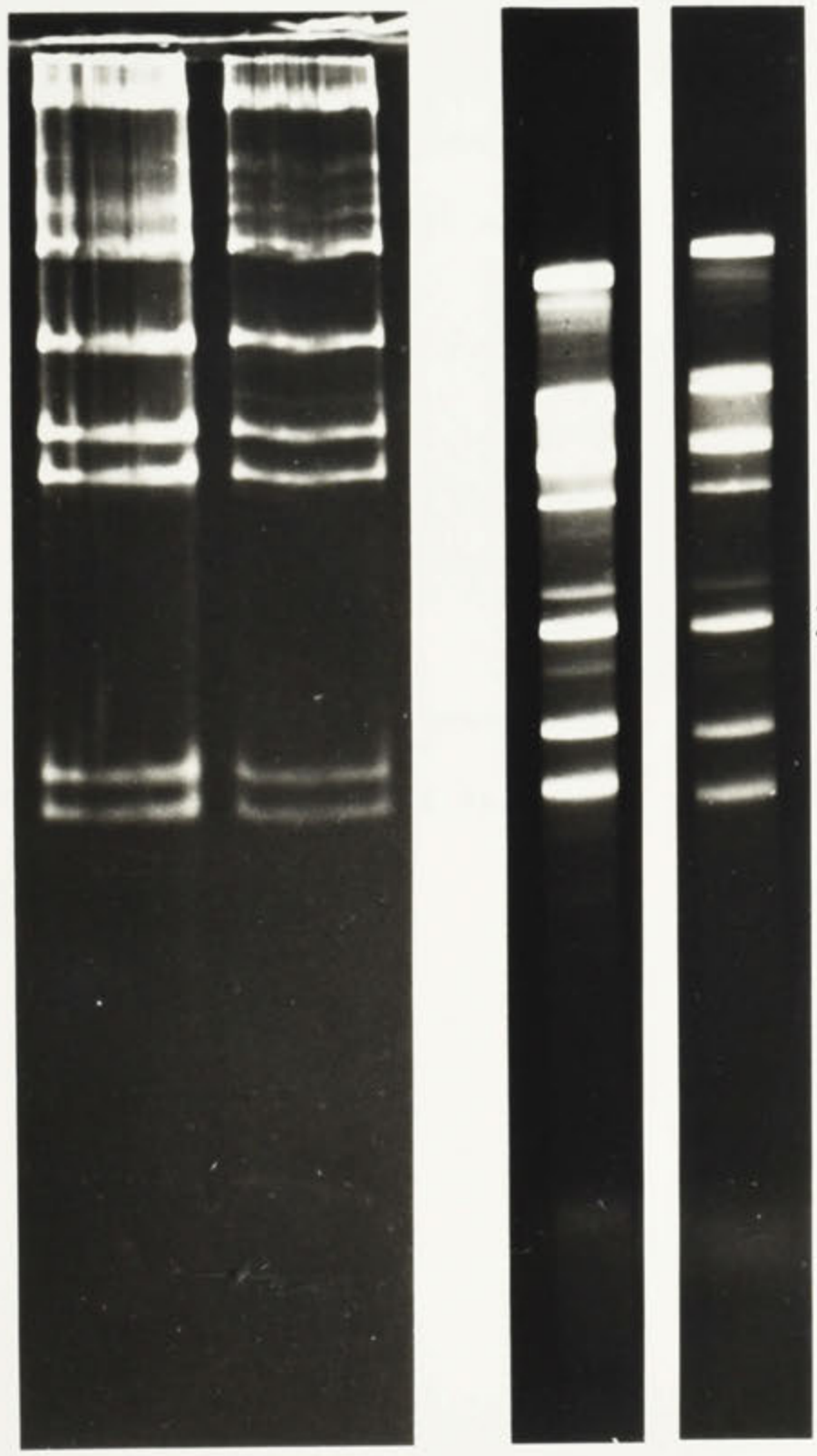

2.1
1.5
1.2
0.3
a b c d

Fig 6

4.1.2. *Sedimentation coefficients*

The New York and Campbell isolates have virions with a sedimentation coefficient of S = 204 (±4). The A.C.T. isolate has smaller sedimentation coefficients of 188S (±5) for the fastest sedimenting component and 116S for the slower fraction.

4.1.3. *Storage of virions*

The infectivity of the purified virion preparations was best retained if they were kept frozen in water with no additives. Storage at 4°C in the presence of thymol phenol blue or sodium azide led to an 80% reduction in infectivity within one month and all infectivity was lost shortly afterwards.

The infectivity of particles frozen in water without additives declined more slowly; the infectivity declined to 80% of the original in about five months (Fig. 7).

4.1.4. *Infectivity assays*

The three isolates, New York, Campbell and A.C.T., developed chlorotic lesions in 8 days in inoculated leaves and had a similar dilution end point of 10^{-4} (Fig. 8).

The mean number of lesions formed increased approximately linearly for each particular inoculum but the replicates were highly variable (Table 4.1).

Table 4.1. Local lesion assay: relationship between lesion number and virion concentration

CaMV concentration	Mean lesions per leaf *	Standard deviation
1	127	± 54.61
10^{-1}	50	± 17.84
10^{-2}	35	± 15.71
10^{-3}	12.75	± 8.7
10^{-4}	0.8	± 1.45

* Each concentration was inoculated on 8 leaves.

The infectivities of the three isolates are compared in Fig. 9. The initial concentration of all inocula was 1 mg of virion/ml of water (O.D. = 7 Shepherd, 1970). The assay plants were selected from a single large group of plants grown in one glasshouse and 8 whole leaves/dilution were inoculated with each virion preparation.

The infectivity of the three isolates in this case did not show appreciable differences but they had different effects on plants. The leaves developing after the inoculation (3rd leaf onward) showed intense systemic symptoms. The New York isolate was the most virulent and A.C.T. the least. Symptoms produced by the isolate New York were more noticeable than those produced by the other two isolates and developed more rapidly; vein clearing in plants infected with isolate New York was visible initially in the third and fourth leaves, the leaves were more distorted, and the roots more deformed. The Campbell isolate caused vein clearing usually visible in the fourth and fifth leaf initially, and

Fig. 7. Comparison of the infectivity of CaMV virions suspensions stored for varying time periods. The infectivity declined to 80% of the original within five months.

Fig. 8. Leaf of turnip cv. Just Right in which the right hand half has been inoculated with CaMV-New York virion suspended in 0.1 M potassium phosphate buffer, pH 7.2, under conditions specified in Materials and Methods. The left hand side was treated under the same conditions with buffer solution only. Chlorotic lesions developed and were counted eight days after inoculation.

Fig. 9. Comparison of the number of local lesions caused by three strains of CaMV (New York - N.Y., Campbell and A.C.T.). The infectivity of the three isolates was not significantly different and all had a similar dilution end-point.

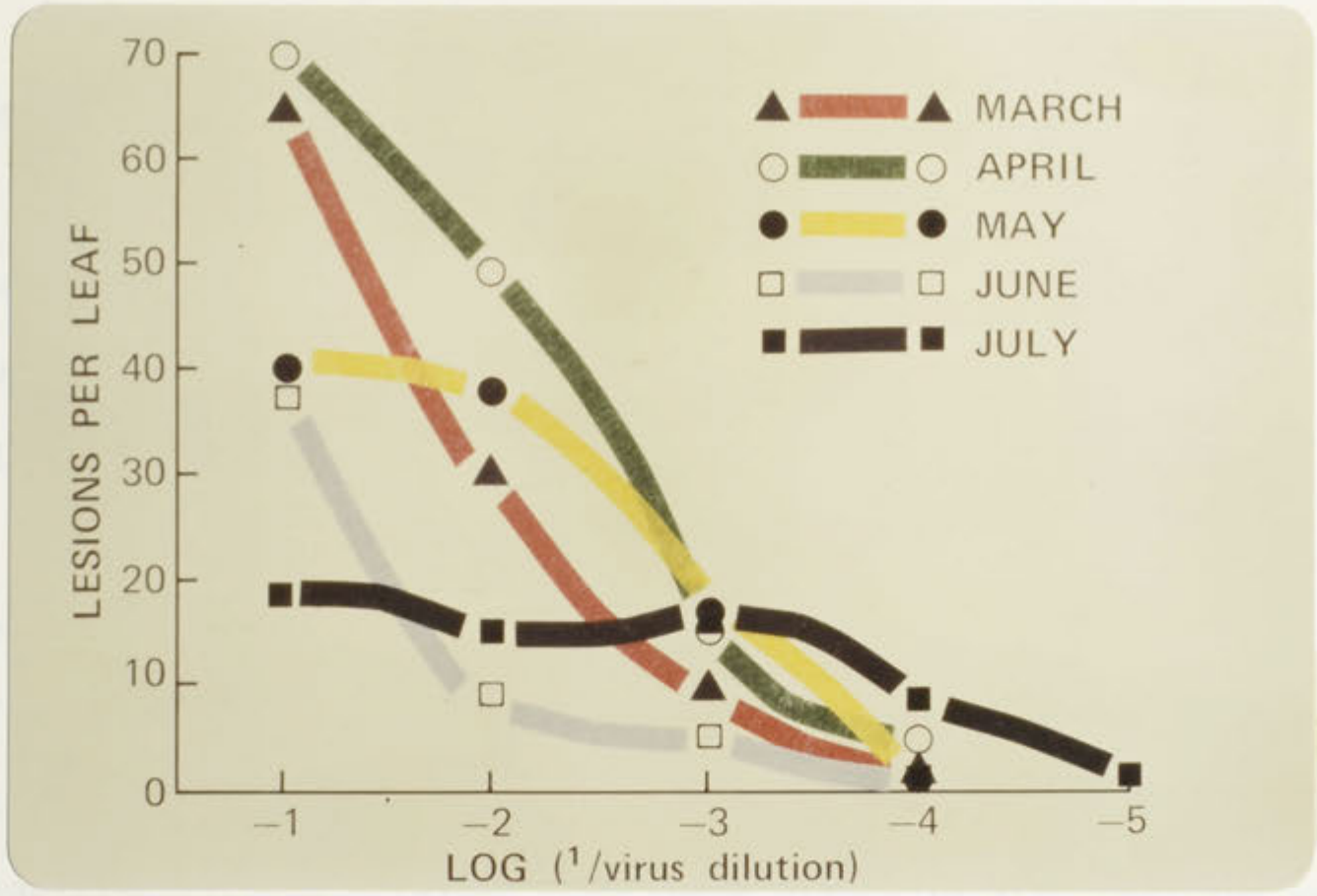


Fig. 7.

Fig. 8.

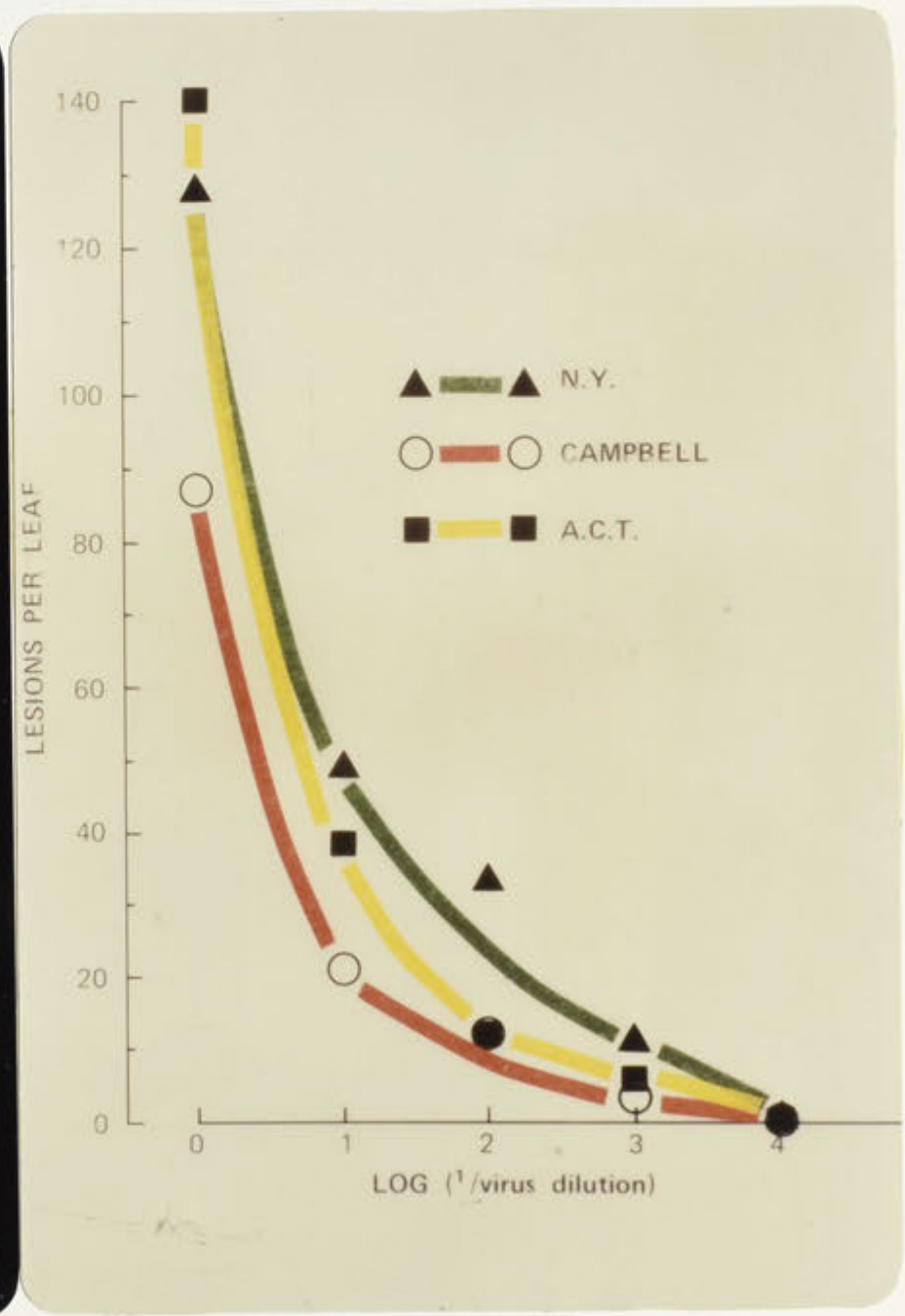


Fig. 9.

chlorosis was less than New York but brighter than A.C.T.

The A.C.T. isolate was the slowest isolate to produce symptoms which never attained the severity of the other two; the first vein clearing to be seen was usually in the fifth and sixth leaves.

A typical example of the different effect of the three isolates on the vegetative growth of turnip is shown by the difference in fresh weight of leaves from 32 infected plants inoculated with each isolate, grown under the same conditions (Table 4.2). The yield of purified virions obtained from these plants is also recorded in Table 4.2.

Table 4.2. The fresh weight of leaves produced by turnip infected with different isolates of CaMV and the subsequent yields of purified virions obtained from them, harvested 3 weeks after infection. Totals for each strain based on 32 plants.

Isolate	Total Fresh Weight (g)	Total Yield of Purified Virions mg
New York	190	3.6
Campbell	220	2.3
A.C.T.	295	1.7

4.1.5. *Host range*

No differences were found in the host ranges of the three strains, and in all the species and cultivars infected, New York always gave the brightest and most severe symptoms, and A.C.T. the mildest symptoms.

All the crucifers inoculated were infected, the most outstanding being turnip cv. Just Right, which was therefore used for virion production and for infectivity tests, and rapeseed cultivar Masowiecki and the race Nantes of *Arabidopsis thaliana*.

The races of *Arabidopsis* that were inoculated showed a range of symptoms. All were susceptible and symptoms took at least 2-3 weeks to appear. The Estland strain inoculated with New York on one occasion gave bright local lesions, and later systemic symptoms; all the infected plants died before flowering, but only rarely were all individuals infected.

In no case did any of the *Nicotiana* species develop symptoms. In particular *N. clevelandii* was investigated in more detail and despite previous reports (Hills and Campbell, 1968) did not show infection. A diverse range of conditions, including temperature, dark treatment and moisture stress were imposed on the plants before and after inoculation.

4.1.6. *Discussion*

Purified preparations of CaMV virions were needed, so they could be used to provide the genomic nucleic acid required for this study.

Virions of all the three strains could be purified by the same procedure but the yields were greatly decreased when frozen material was used. This made it necessary to grow a continuous supply of fresh plant material throughout the duration of the work reported in this thesis.

Once the virions were isolated, the most appropriate way to store them had to be found. Losses of infectivity of virion preparations during storage have been reported (Hull and Shepherd,

1977) and of the methods tried, storing the virions frozen in water proved to be most satisfactory.

A convenient and reliable method for measuring infectivity was required as *Datura stramonium*, which had been reported to be a local lesion host (Lung and Pirone, 1972) gave erratic results. Turnip, cv. Just Right proved to be a reliable local lesion host. The production of local lesions in turnip mainly depended on the age of the seedlings when inoculated. If fully developed leaves were inoculated no lesions were formed, or if the leaves were too young at the moment of inoculation lesions were only formed in the distal parts of the leaves. Darkening the plants after inoculation accentuated the chlorotic lesions making counting easier but did not alter the number of lesions formed. At different times of the year different numbers of lesions were produced, most in spring, and fewest in winter. Since the glasshouse had a more or less constant temperature throughout the year, this effect was probably due to the effect of light intensity or photoperiodism.

The number of lesions was consistently linearly related to the concentration of virions for all the strains, but not different enough to allow to distinguish between the strains. The systemic symptoms in plants infected with each of the three CaMV isolates were distinguishable from each other. There was also a relation between the degree of infection expressed as severity of symptoms, and the yield of purified virions.

Since the severity of symptoms increased with time, the mildest isolate, A.C.T. was usually kept longer in the glasshouse in order to compensate for the poor yield of virions. However, as a consequence, a greater proportion of slowly sedimenting virions was obtained.

The isolate A.C.T. had a smaller sedimentation coefficient than New York or Campbell, and gave two bands in sucrose gradients. Since the morphology of virions of both bands, as observed in the electron microscope, seemed identical and the DNA extracted from them was homologous it has to be assumed that differences in the protein of the virions was responsible for this phenomenon. Al Ani *et al.* (1979) using a variant of the strain Cabbage B, found that virions purified from fresh leaves gave rise to an asymmetrical peak with a shoulder on sucrose gradient, or even to a second slow-migrating peak, and attributed this to partial proteolysis of the proteins. Preparations of virions obtained from frozen material did not present this problem, and he attributed this to inactivation of some plant proteases on freezing.

In the three strains of CaMV used in this study, freezing the infected leaves decreased the yield of the virions, especially in those of the A.C.T. isolate, where no virions were recovered from frozen leaves, so no comparison between virions from fresh leaves and frozen leaves was possible.

But proteolysis of the virions could offer a possible explanation for the anomalous sedimentation behaviour of A.C.T. virions. The appearance of asymmetry in the virion band in sucrose gradients was related to the age of the plants from which the virions were isolated, with the oldest plants (5-6 weeks after inoculation) giving the two clearest peaks. This property must be due to some virus/host factor, not solely to a host factor, since all the strains were propagated in the same host under the same conditions. It might also indicate some relation between this factor and the lower infectivity of the strain A.C.T.

Freezing decreased the yield of virions of all strains but the isolate New York always gave the highest yield, hence loss caused by freezing was less important than with the A.C.T. isolate as any loss would result in the virions being below a detectable level.

Arabidopsis spp. proved to be a good host for the three isolates of CaMV. The fact that not always 100% of the inoculated plants developed symptoms was most probably due to defects in the inoculation procedure. The leaves of *Arabidopsis* at the time of inoculation had an average length of only 2-4 mm. It was quite difficult to rub the inoculum onto the leaves and at the same time not to cause extensive damage which could preclude the establishment of infection.

4.2. PROPERTIES OF THE GENOME DNA OF CAULIFLOWER MOSAIC VIRUS

4.2.1. *Nucleic acid purification*

The yield of viral DNA from the three isolates was variable. The most complete recovery of DNA was from virions of New York; up to 90% was obtained from virus samples, assuming a DNA content of 17% (Shepherd, 1976); less was recovered from Campbell virions and least from A.C.T. virions.

The three isolates yielded a heterogeneous population of DNA consisting of circular and linear molecules. The ratio between circular and linear molecules differed for each of the three isolates. DNA isolated from recently purified virions of New York consisted mainly of circular molecules with 10% or less linear. Similarly, Campbell DNA was mainly circular when just isolated, but the proportion of linear molecules increased with ageing more

rapidly than in New York. About half the A.C.T. DNA was linear when isolated (Fig. 10, 1a, 1b).

The proportion of circular to linear molecules was estimated from agarose gels by comparing the intensity of fluorescence in the appropriate bands (Fig. 10 a,b). The heterogeneity present in the three isolates agreed with observations made by Hull and Shepherd (1977) and Civerolo and Lawson (1978) who found a positive correlation between the infectivity of CaMV and the proportion of circular molecules. Isolate New York consistently produced a greater proportion of circular molecules and was the most virulent isolate. On storage, the proportion of linear molecules increased and the infectivity of the DNA decreased.

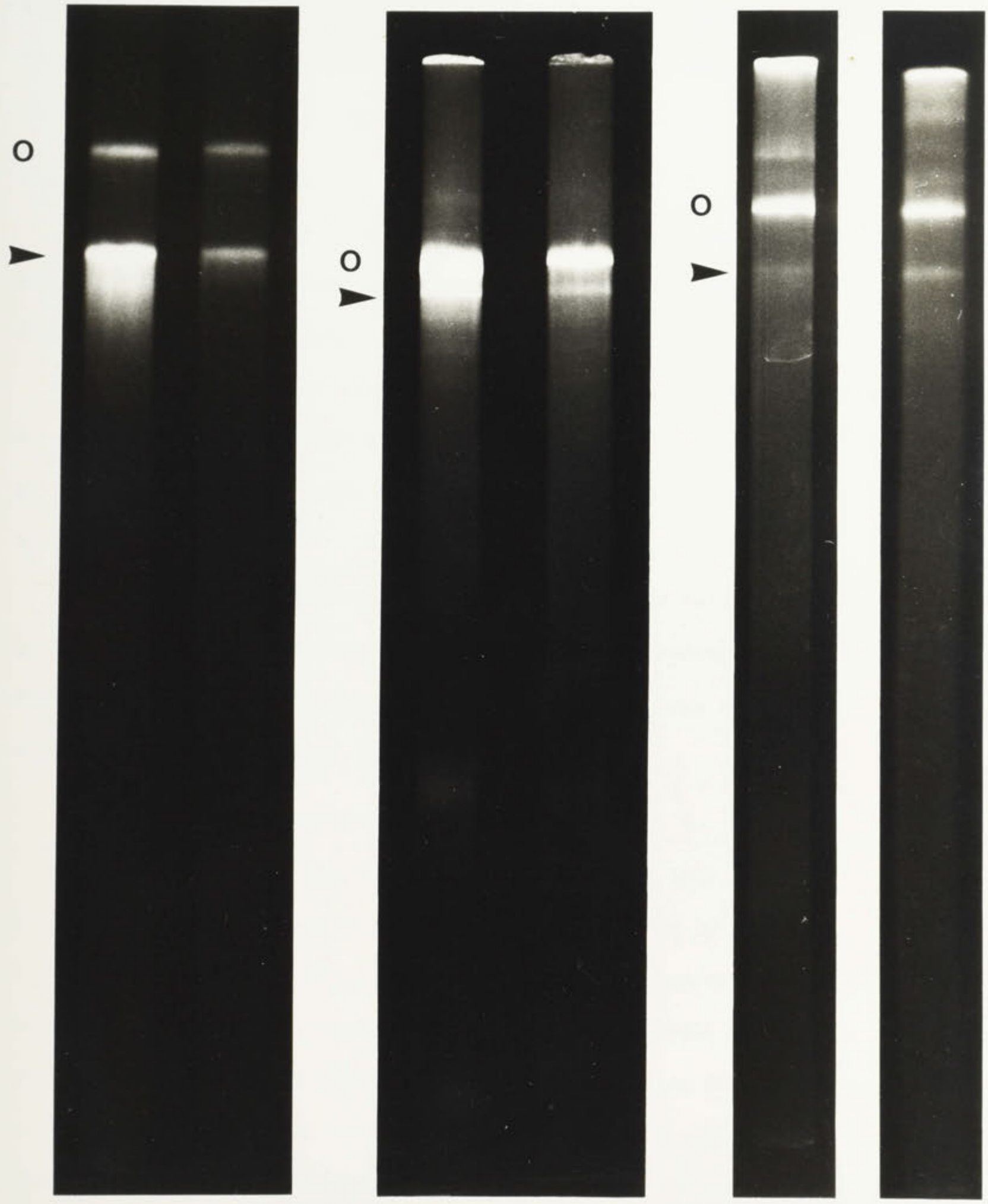
After 6 weeks in storage the infectivity of the DNA was low; no more than half of the test plants were infected at higher concentrations (10, 20 or 40 µg DNA in 0.5 ml).

In no case did all the DNA molecules linearise. At most, half changed from circular to linear, but the infectivity of DNA after 6 weeks was low and erratic. Because of this, whenever DNA was needed for inoculation, fresh virions were isolated and the DNA used within 5 weeks.

The Campbell isolate behaved like New York, but a greater concentration of its DNA was required to obtain the same infectivity as preparations of New York DNA. Also the Campbell DNA "aged" more quickly, with most of the infectivity lost within 5 weeks. This limited to 4 weeks the period during which Campbell DNA could be used.

Fig. 10. Agarose gels (0.8%) into which untreated genomic DNA of the different CaMV isolates had been electrophoresed, illustrating that the DNA is a heterogeneous mix of linear and circular molecules.

1a and 1b represent samples of A.C.T. DNA showing a large proportion of linear molecules (►). 2a and 2b correspond to New York DNA samples containing mainly circular molecules (O). 3a and 3b show samples of Campbell DNA containing different proportions of circular (O) and linear molecules (►). In these last two gels slower migrating bands corresponding to "twisted" molecules can be seen. The apparent difference in relative migration rate of circular and linear molecules for A.C.T. (tracks 1a and 1b) as compared with New York (track 2a and 2b) result from different times being used during electrophoresis.


O
O
O
1a
1b
2a
2b
3a
3b

The infectivity of A.C.T. DNA was always very small and never more than half that of the other isolates and was also very variable. Because of these properties, this isolate was never used in studies where infectivity was being tested.

4.2.2. *Restriction mapping*

An endonuclease restriction map was constructed for the Campbell isolate, and this provided a standard with which the other two isolates could be compared.

First the molecular weight of the native linear DNA was estimated by electrophoresis in 0.8% agarose gels and found to be 4.8×10^6 daltons by comparison with suitable markers (See Materials and Methods Section 3.5.3.).

The first stage in constructing the map was to determine the number of fragments generated by each restriction endonuclease. Following that, the cleavage point for each enzyme was determined with respect to the Eco R1 sites.

4.2.2.1. Eco R1 endonuclease

Campbell DNA digested with Eco R1 was cleaved into 3 main fragments with molecular weights of 2.1×10^6, 1.5×10^6 and 1.2×10^6 daltons (Track a, Fig. 11). A fourth fragment of molecular weight 0.3×10^6 daltons was also found but in variable amounts depending on the age of the DNA, which suggested that it might be a product of hydrolysis of spontaneously formed linear molecules. The sum of the molecular weights of the three main fragments was 4.8×10^6 daltons which is the most commonly reported molecular weight of the genome (Shepherd *et al.*, 1970; Russell *et al.*, 1971). If it is assumed that the small molecular weight fragment resulted from the presence of a fourth Eco R1 site then the estimated molecular weight of the genome is 5.1×10^6 daltons.

The possibility that the small fragment (0.3*) arose from the linearization of the genome, at a point 0.3 x 10^6 daltons distant to one of the Eco R1 sites was examined by analysis of the CaMV/pmB 9 hybrid molecule provided by Dr. J. Langridge. This hybrid molecule had been constructed by inserting CaMV DNA in the bacterial plasmid using a Bam I site. After cloning, the CaMV portion of this hybrid was excised by digestion with Bam I and the complementary staggered ends ligated (Materials and Methods Section 3.5.9.). This gave a population entirely of circular CaMV molecules. Digestion of these circular forms also gave the 0.3 fragment (Fig. 11, Track K), indicating that this fragment was part of the molecule and not only an artifact due to linearization.

A more accurate estimate of the size of the Eco R1 fragments was obtained by coelectrophoresis of the Eco R1 digest of CaMV DNA with an Eco R1 digest of λ DNA (Fig. 11, Track e-j). Both linear and circular molecules of CaMV DNA were present in the digested samples. This confirmed that the molecular weights of the Eco R1 fragments were 2.1 x 10^6 1.5 x 10^6, 1.2 x 10^6 and 0.3 x 10^6 daltons. In the native DNA the band corresponding to the linear molecules in the sample had a mobility corresponding to a molecular weight of 4.8 x 10^6 daltons. This disparity in molecular weights between linear molecules and the sum of the linear excised fragments will be discussed later in this thesis (Section 4.2.3.).

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* Throughout this chapter the fragments are referred to by their sizes in units of 10^6 daltons, thus an 0.3 fragment refers to one with a molecular weight of 0.3 x 10^6 daltons.

Fig. 11. Electrophoretic analysis of CaMV genomic DNA digested with Eco R1 restriction endonuclease.

(a) Campbell DNA after complete digestion with Eco R1.

(b, c, d) Campbell DNA partially digested with Eco R1 for 20, 10 and 5 min, respectively. The molecular weights on the right side of track d represent the most likely interpretation of the fragmentation patterns. The molecular weights (x 10^{-6} daltons) of the bands are presented in the left hand side of track b.

(e) Untreated New York DNA. The arrow indicates the band containing the circular molecules present in the population. The linear molecules migrate at a rate corresponding to a molecular weight of 4.8 x 10^6 daltons.

(f) New York DNA digested with Eco R1 coelectrophoresed with λ DNA digested with Eco R1. The sizes of the CaMV fragments are shown in the left hand side. CaMV fragments identified by a white spot.

(g) Untreated Campbell DNA

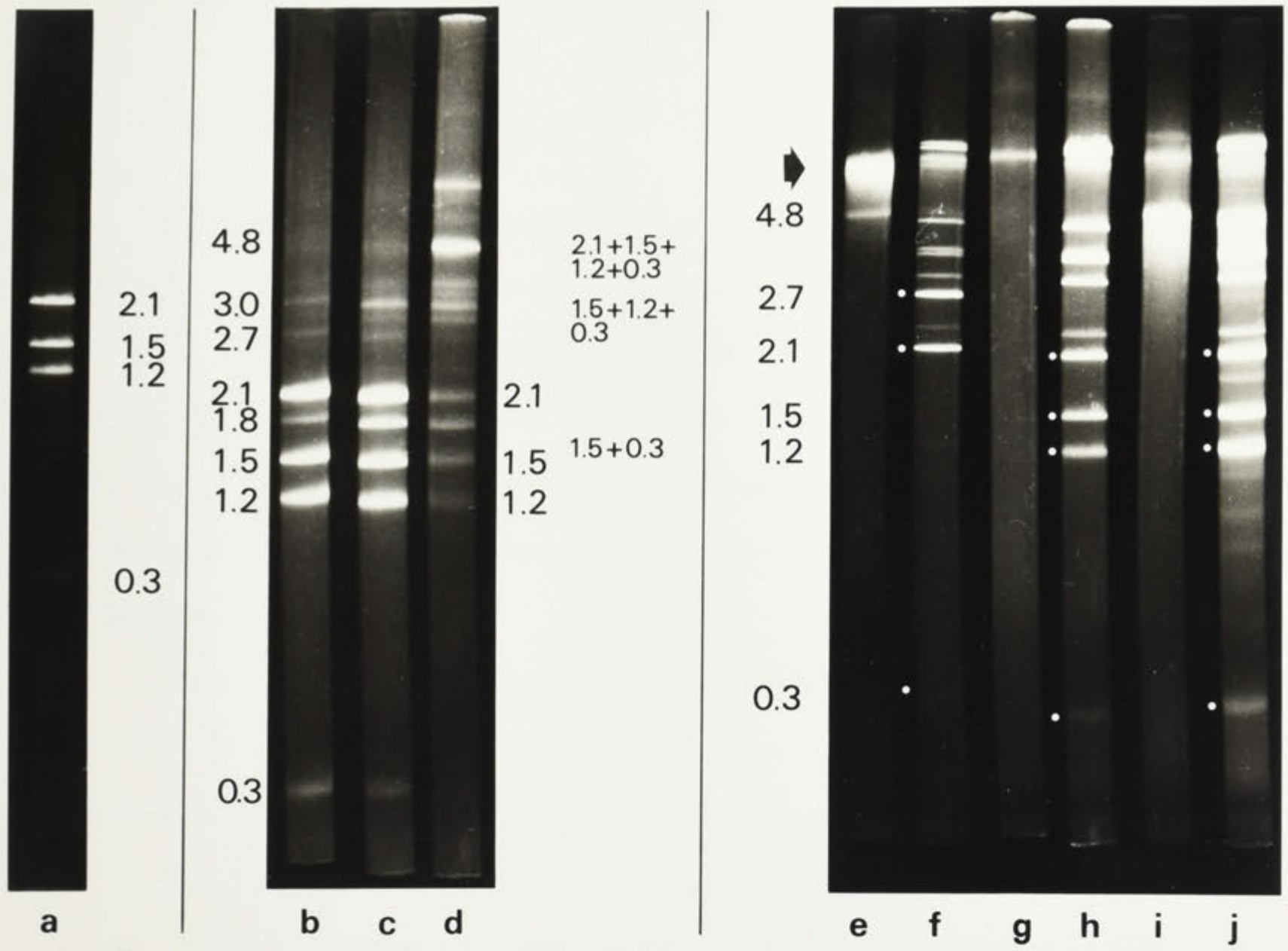
(h) Campbell DNA digested with Eco R1, and electrophoresed with Eco R1 digested λ DNA.

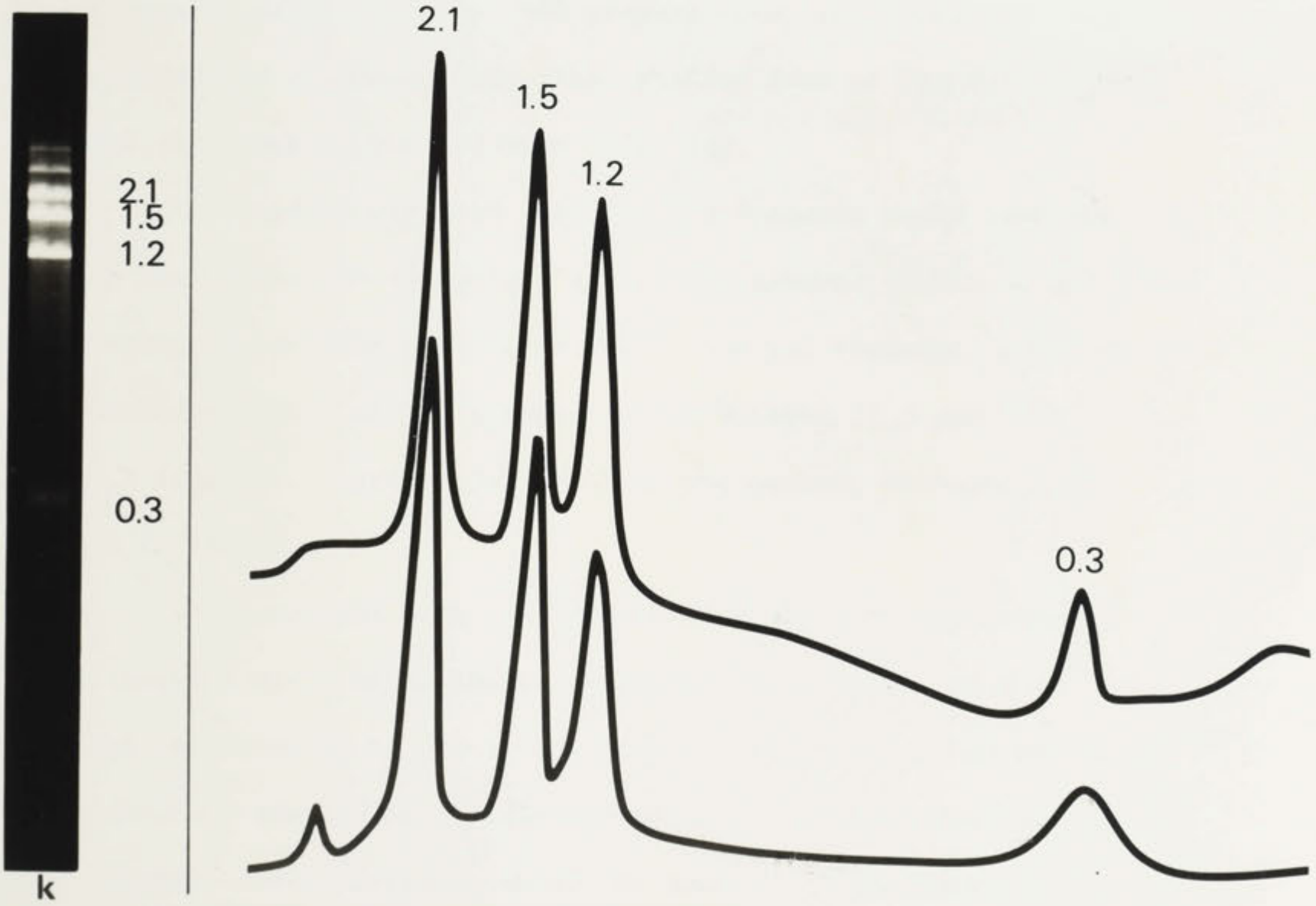
(i) Untreated A.C.T. DNA.

(j) A.C.T. DNA and λ DNA after digestion with Eco R1.

(k) Cloned CaMV DNA after digestion with Eco R1.

The densitograms in the lower right corner represent the changes in the proportions of fragments of Campbell DNA after Eco R1 digestion. The changes in the relative proportions of the upper and lower traces reflect differing proportions of linear molecules in the native DNA population.


a
2.1
1.5
1.2
0.3
b c d
4.8
3.0
2.7
2.1
1.8
1.5
1.2
0.3
2.1+1.5+
1.2+0.3
1.5+1.2+
0.3
2.1
1.5
1.2
1.5+0.3
e f g h i j
4.8
2.7
2.1
1.5
1.2
0.3


k
2.1
1.5
1.2
0.3
2.1
1.5
1.2
0.3

The order of the Eco R1 fragments within the molecule was determined by electrophoresis of partially digested Campbell DNA. Samples were incubated at room temperature and aliquots taken after 5, 10, 20 and 30 min. After 30 min, digestion was complete (Track a, Fig. 11). The results of partial digestion and the most likely interpretation of the fragmentation patterns are presented in Fig. 11, Track b-c-d).

This shows that the most probable orientation of the fragments is 2.1, 0.3, 1.5 and 1.2. The 2.4 fragment that would unambiguously define the position of the 0.3 fragment was never found.

The three larger bands were obtained in equimolar proportions from freshly isolated DNA (mainly circular molecules) with the 0.3 fragment hardly visible. DNA preparations that contained larger proportions of linear molecules, yielded more of the 0.3 fragment and also more of the 1.2 band (Fig. 11).

It would be expected that the 0.3 fragment would comprise 5.8% of the DNA in the analytical gels. The amounts found, at different times, varied between 6.9% to 9.2%. The 1.2 fragment, which should represent 23.5% of the genome, varied between 27.5 and 33%. The 1.5 fragment, representing 29.4% of the genome, decreased from 29.6% to 27%.

This suggests that the breakage enabling the molecules to linearize might be in the 1.5 fragment, at a point 0.3×10^6 from the end adjacent to the 1.2 fragment. Thus, in samples having an increased proportion of linear molecules, the proportion of the 1.5 fragment would decrease, while the relative proportions of the 1.2 and 0.3 fragments would increase. On this basis it would also be expected that the 0.3 fragment should have sequence homology with

the 1.5 fragment. These expectations were tested and the results and implications derived from them will be discussed later in this thesis (Section 4.3.11.).

The enzyme sites were mapped with respect to the Eco R1 sites and that between the 0.3 and 1.5 fragments I call the origin (Site 1). Then, going clockwise around the map, the site between the 1.5 and 1.2 fragment is site 2, that between the 1.2 and 2.1 fragment site 3. Finally the site between the 2.1 and 0.3 fragment is the 4th site.

4.2.2.2. Sal I endonuclease

Sal I cleaves the CaMV genome at one point and gives one major band with a molecular weight of 4.8×10^6 daltons. This corresponds to linear molecules generated by a single Sal I site in the circular molecule (Track a, Fig. 12). Two minor bands of molecular weight 2.9×10^6 daltons and 2.1×10^6 daltons were also present. The sum of these two fragments is close to the molecular weight of the complete molecule (4.8×10^6 or 5.1×10^6 considering the Eco R1 digestion) suggesting that they come from the linear molecules present in the sample.

When CaMV DNA was double digested with both Sal I and Eco R1 (Track b, Fig. 12) the 2.1 Eco R1 band disappeared, the 1.5 band became a doublet and a new 0.6 band appeared. This indicated that the Sal I site was present in the 2.1 Eco R1 fragment, cleaving it into two fragments, a 1.5 fragment that would run together with the R1 fragment of the same size, and a 0.6 fragment.

To further verify this point, the 2.1 Eco R1 band was isolated by the freeze-squeeze method and digested with Sal I. Again the 2.1 Eco R1 band was cleaved into a 1.5 and 0.6×10^6 fragment (Fig. 12, track f and g).

Fig. 12. Electrophoretic analysis of Campbell DNA after restriction with Sal I endonuclease. (a)

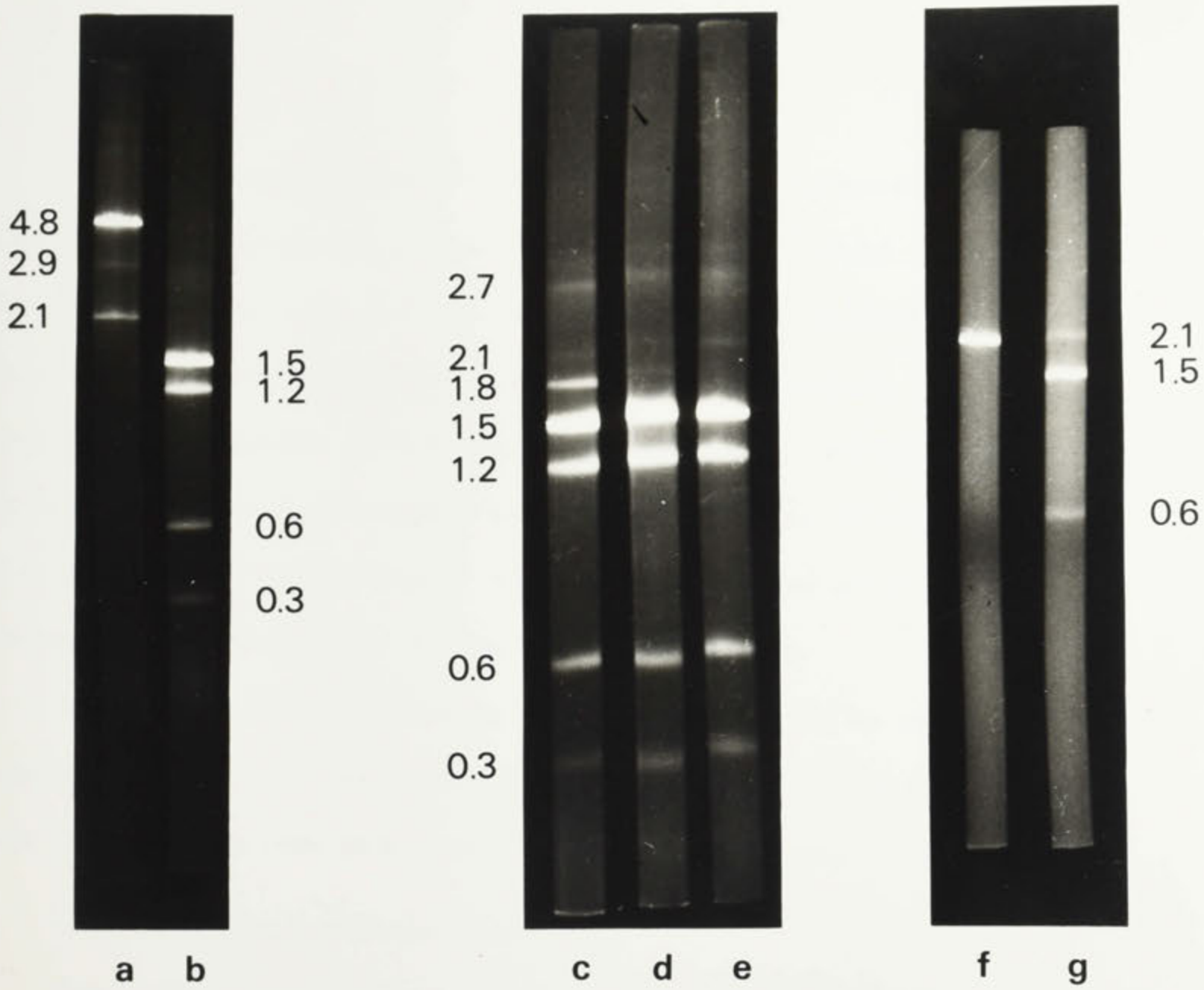
(b) Electrophoretic analysis of Campbell DNA digested with both Eco R1 and Sal I restriction endonucleases. The densitogram at the bottom was from (b).

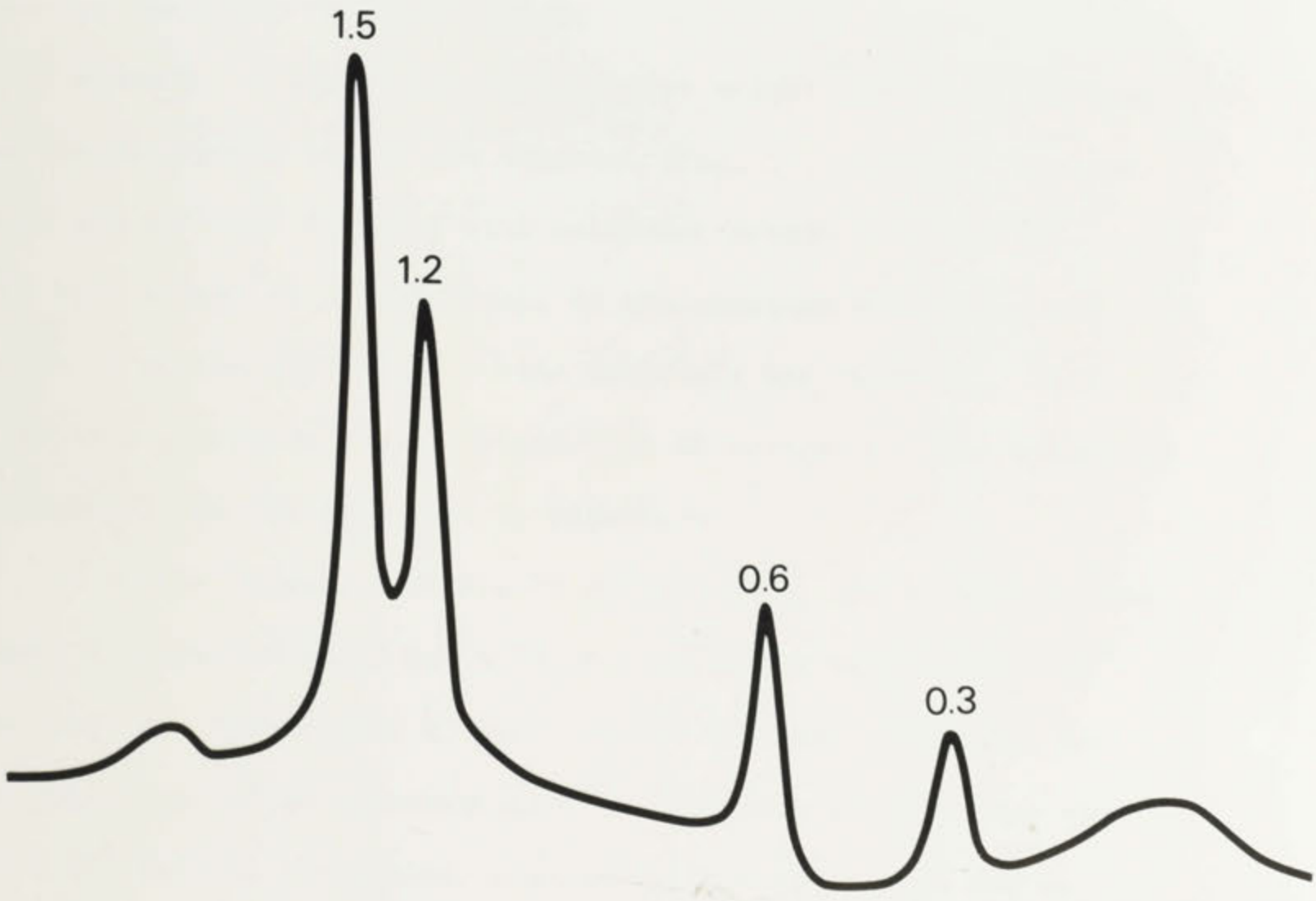
(c, d, e) Campbell DNA completely digested with Sal I and partially digested with Eco R1 for 20, 10 and 5 min, respectively.

(f) Electrophoresis of the 2.1 Eco R1 fragment isolated using the freeze-squeeze method.

(g) 2.1 Eco R1 fragment isolated and subsequently digested with Sal I.

Sizes indicated are the molecular weights x 10^{-6} daltons.


4.8
2.9
2.1
1.5
1.2
0.6
0.3
2.7
2.1
1.8
1.5
1.2
0.6
0.3
2.1
1.5
0.6
a
b
c
d
e
f
g


1.5
1.2
0.6
0.3

The cleavage site could have two possible orientations within the 2.1 Eco R1 fragment. First, the 0.6 fragment could be adjacent to the 4th Eco R1 site and the 1.5 to the 3rd Eco R1 site. A molecule with this orientation, when partially digested with Eco R1, should give fragments of 0.9×10^6 and 2.7×10^6. In the alternative orientation , the 0.6 fragment could be adjacent to the 3rd Eco R1 site and the 1.5 to the Eco R1 site 4. Thus a band of 1.8×10^6 daltons should be present in the partial digestion.

When completely digested by Sal I and partially by Eco R1, a band at the 1.8×10^6 position was found (Fig. 12, track c,d,e,) confirming the second orientation as the correct one.

4.2.2.3. Bam I endonuclease

Bam I also cleaved the molecule at one point, linearizing the circular DNA. The molecular weight of this linear molecule was 4.8×10^6 daltons. A minor band of molecular weight 3.9×10^6 daltons was always present in all the digests, (Fig. 13, track a), however, there was no other fragment with molecular weight of 1.6×10^6. With this enzyme it was difficult to get complete digestion, and excess of enzyme applied at 2 time intervals was required. Even after this procedure a small proportion of native circular molecules of Campbell DNA was resistant to digestion.

In double digests with Eco R1 endonuclease, the 1.2 Eco R1 band almost disappeared and a new band of a molecular weight 0.9×10^6 was visible. The 0.3 Eco R1 band did not increase in density but the region in the gel corresponding to molecular weights less than 0.2×10^6 daltons fluoresced, suggesting that there were one or two bands of less than 0.2×10^6 daltons which were beyond the resolution of the system (Fig. 13, track b).

The 1.2 Eco R1 band was isolated by the freeze-squeeze method and digested with Bam I. A clear band of 0.9 x 10^6 daltons was present, but no other bands were visible in the low molecular weight region of the gel (Fig. 13, track e,f,).

The orientation of the Bam I site was determined by its relative distance to the previously mapped Sal I site.

If all the population of CaMV DNA were circular and there was one Sal I site and one Bam I site the possible result of a double digestion with these two enzymes would be as follows.

(a) If the 0.9 fragment generated by Bam I in the 1.2 Eco R1 band is adjacent to the Eco R1 site 2, two bands with molecular weights of 1.5 x 10^6 and 3.6 x 10^6 daltons should be present.

(b) If the 0.9 Bam I fragment is adjacent to the Eco R1 site 2, then double digestion with Bam I and Sal I would result in a fragment with a molecular weight of 0.9 x 10^6 daltons and another with a molecular weight of 4.2 x 10^6 daltons.

The 0.9 Bam I fragment should originate from the DNA population that has only one Bam I site as suggested by the linearization of the molecule. However, if a small percentage of the population has more than one Bam I site, as the absence of a 0.3 fragment in the Eco R1 Bam I double digest might indicate, the double digest with Sal I should show it. In this case, more than two bands should be obtained, and the molecular weights of the extra bands should not differ by more than 0.1 or 0.2 x 10^6 daltons. Only fragments of this size can explain the fluorescence seen in regions of the gel where fragments smaller than the 0.3 Eco R1 fragment could be seen in the Eco R1-Bam I double digest, and which would complement the missing 0.9 fragment.

Fig. 13. Electrophoretic analysis of Campbell DNA digested with Bam I restriction endonuclease. (a)

(b) Campbell DNA digested with both Eco R1 and Bam I restriction endonuclease. The densitogram at the bottom was obtained from gel (b). The black dot identifies the Eco R1 1.2 fragment which is cleaved by Bam I to yield the 0.9 fragment.

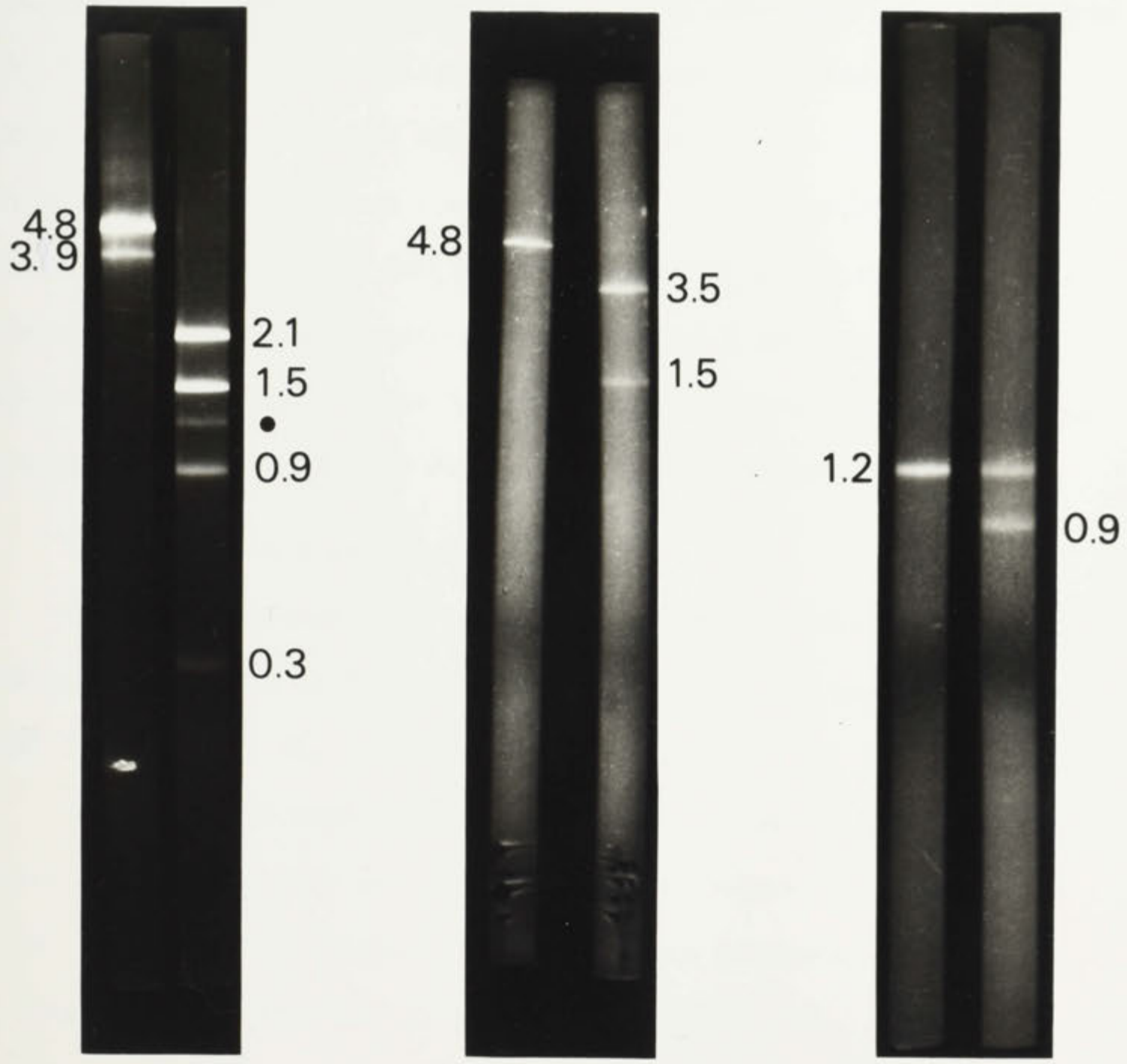
(c) Electrophoresis of molecules linearized by digestion with Sal I and isolated using the freeze-squeeze method.

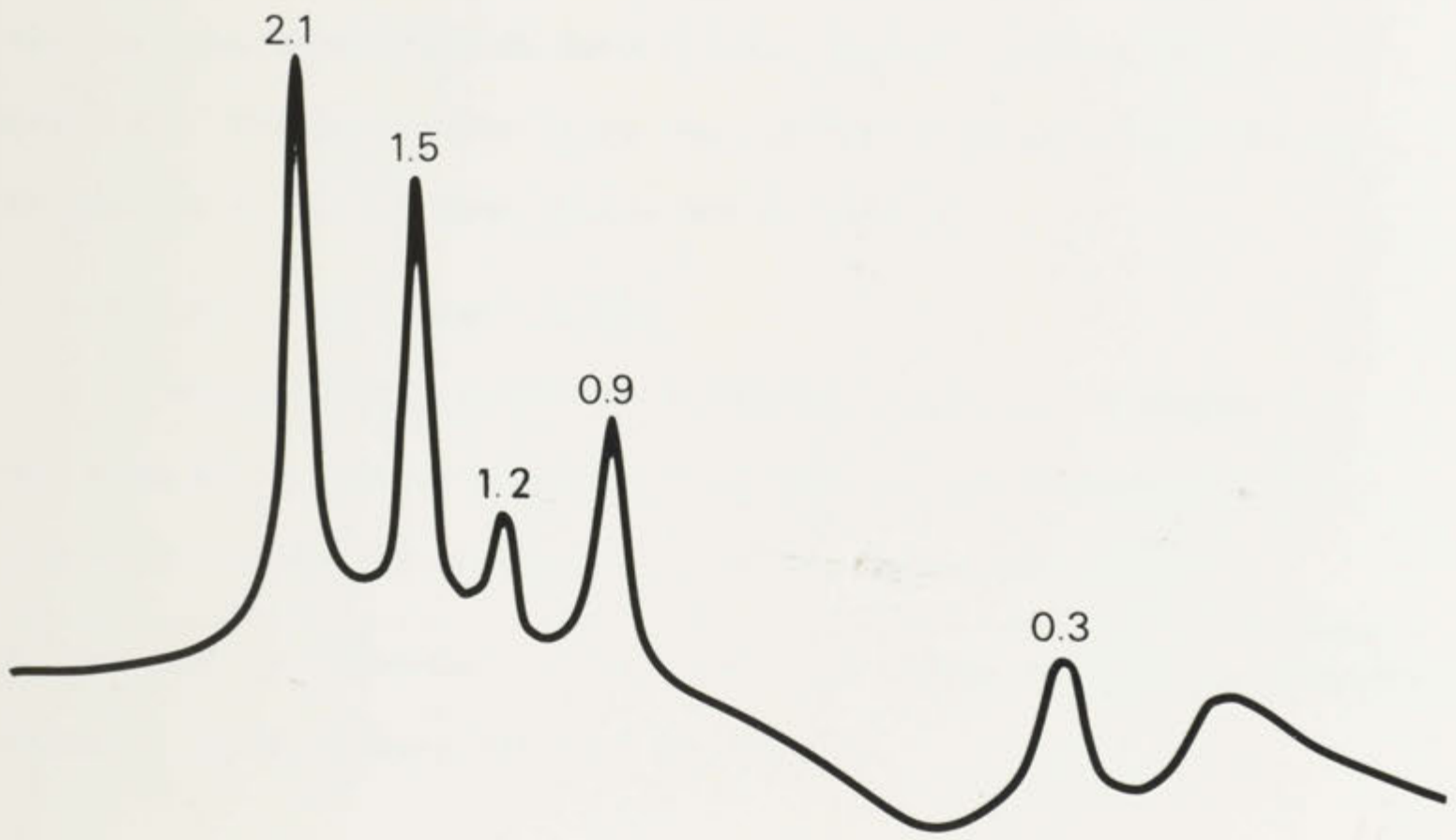
(d) Isolated linear molecules subsequently digested with Bam I.

(e) Electrophoresis of the 1.2 Eco R1 fragment after isolation using the freeze-squeeze method.

(f) 1.2 Eco R1 fragment isolated and subsequently digested with Bam I.

Molecular weights x 10^{-6} daltons.


4.8
3.9
2.1
1.5
0.9
0.3
4.8
3.5
1.5
1.2
0.9
a
b
c
d
e
f


2.1
1.5
1.2
0.9
0.3

To avoid confusion with fragments originating from the linear population the DNA was digested with Sal I. The main band corresponding to the molecules linearized by Sal I were isolated by the freeze-squeeze method and digested with Bam I (Fig. 13, track c,d). Only two clear bands were found and these had molecular weights of 1.5×10^6 and 3.5×10^6 daltons. Consequently, the 0.9×10^6 fragment obtained by digesting with both Bam I and Eco R1 maps is adjacent to the Eco R1 site 3. This also confirms that the circular molecules of Campbell CaMV have only one Bam I site.

4.2.2.4. Xho I endonuclease

This endonuclease also cleaved at one site in the Campbell DNA molecule, although it did not completely digest the genome of this CaMV strain (Fig. 14, track a).

When digested with both Xho I and Eco R1 the 1.2 Eco R1 band decreased in intensity and two new bands of molecular weight 0.76×10^6 and 0.44×10^6 daltons appeared, which indicates that the Xho I site is within this Eco R1 band (Track b, Fig. 14). This was confirmed by isolating the 1.2 Eco R1 band and digesting it with Xho I endonuclease (Fig. 14, track d). The orientation of the fragment was determined as before using partial digests (Fig 14, track c). The Xho I site is in the 1.2 Eco R1 fragment and closer to the Eco R1 site 3 than to the Eco R1 site 2.

4.2.2.5. Xba I endonuclease

It was difficult to obtain a complete digestion of Campbell DNA with Xba I, and as specified in Materials and Methods (Section 3.5.3) 6-7 units* of enzyme per µg of DNA were used. When a

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* One unit is equivalent to the amount of enzyme required to completely digest 1 µg of λ phage DNA in 1 hr. at 37°C.

Fig. 14. Electrophoretic analysis of Campbell DNA digested with Xho I restriction endonuclease (a).

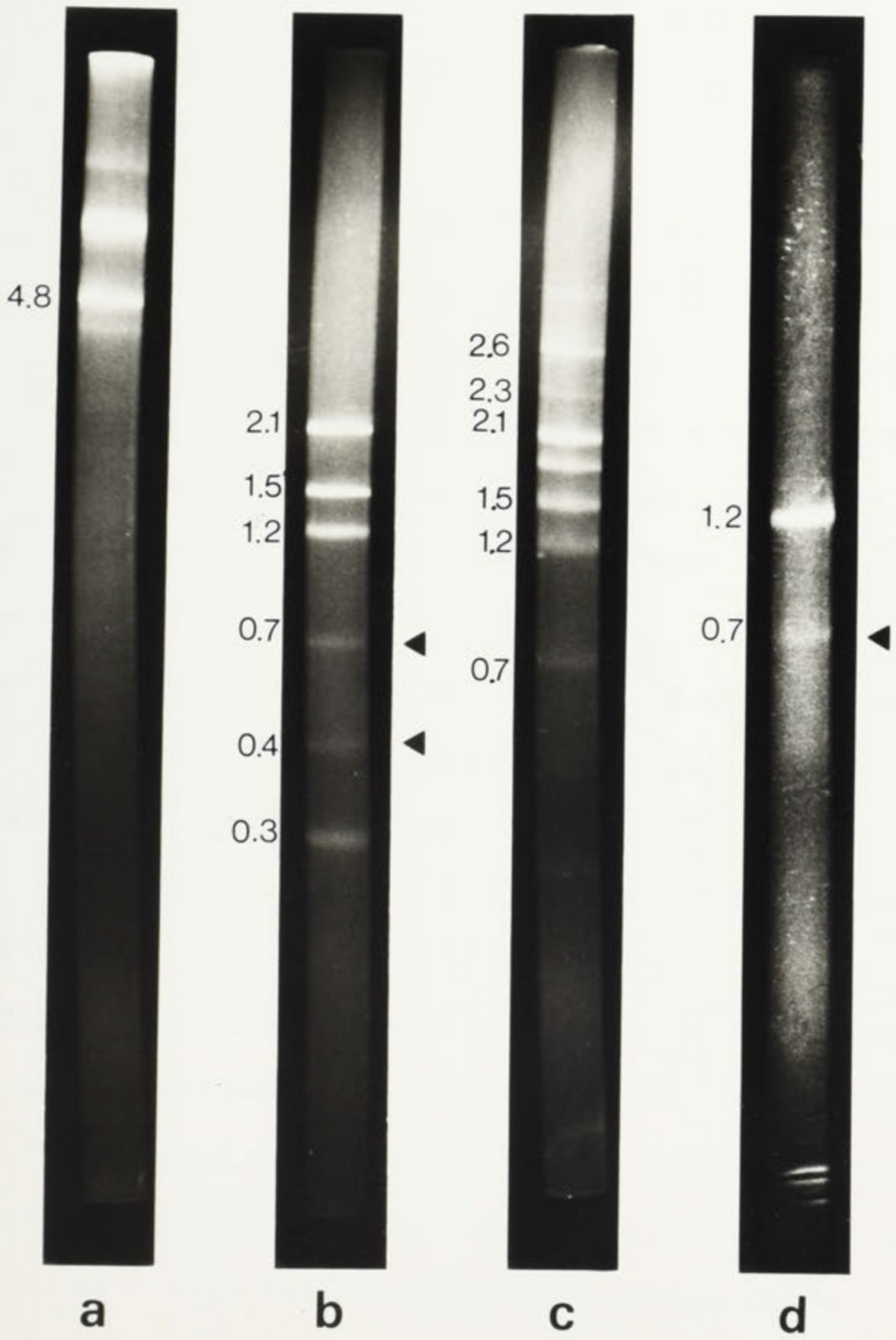
(b) Campbell DNA digested with both Xho I and Eco R1 restriction endonucleases.

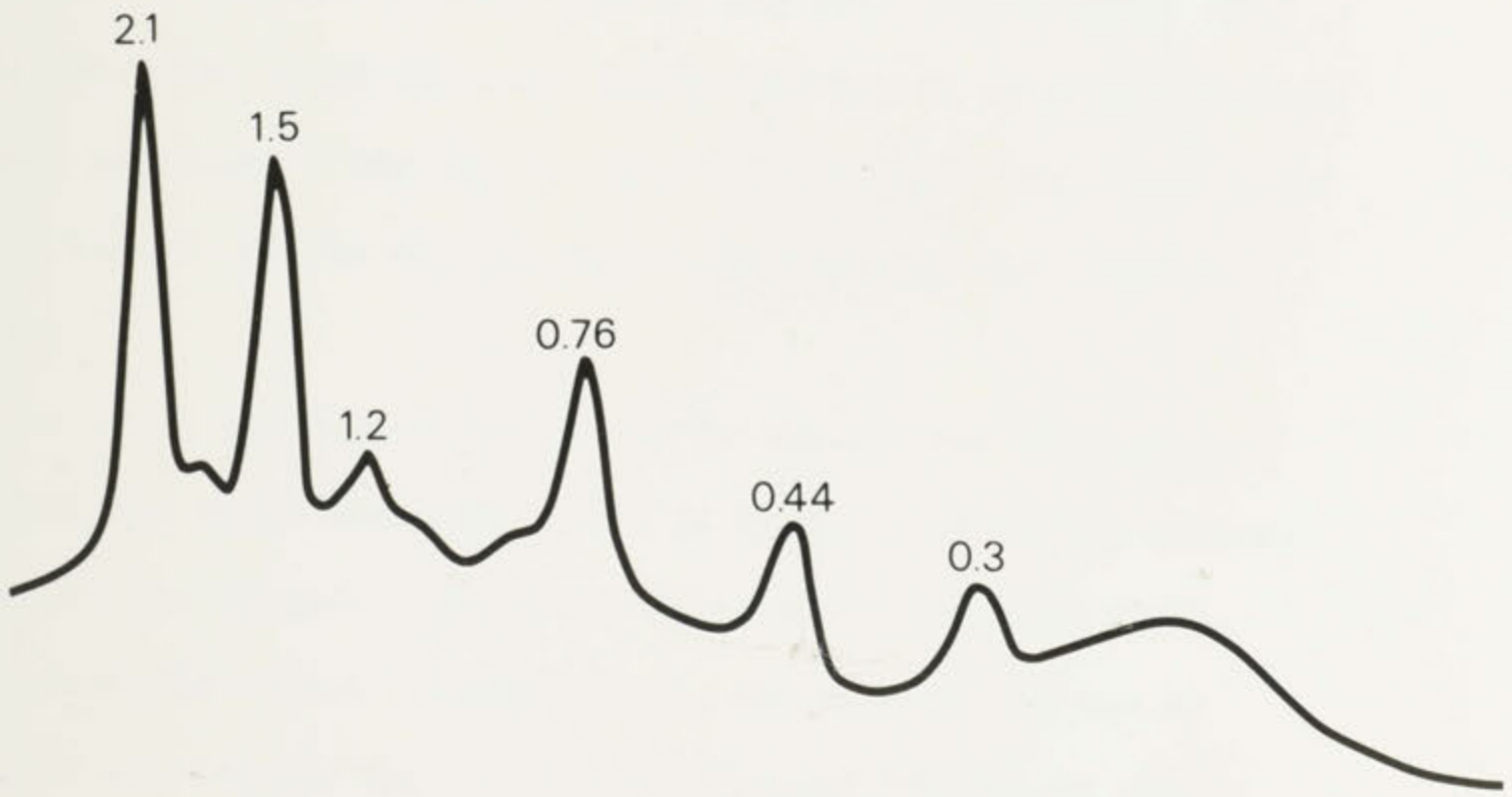
(c) Partial digestion of Campbell DNA with Xho I and Eco R1.

(d) Electrophoresis of the 1.2 Eco R1 fragment isolated and further digested with Xho I.

The densitogram corresponded to Campbell DNA double digested with both Xho I and Eco R1 (track b). Molecular weight x 10^{-6}.

Arrows refer to Xho I bands.


4.8
2.1
1.5
1.2
0.7
0.4
0.3
2.6
2.3
2.1
1.5
1.2
0.7
1.2
0.7
a
b
c
d


2.1
1.5
1.2
0.76
0.44
0.3

complete digestion was obtained, four major bands were obtained with molecular weights of 2.5, 1.65, 0.5 and 0.5 x 10^6 daltons (Fig. 15, track e). A minor band of 2.1 x 10^6 daltons was also present. Addition of more enzyme or prolonged incubation did not alter the relative intensity of this band suggesting that it probably originated from the linear molecules present in the native DNA population.

The genome digested with both Xba I and Eco R1 contained neither the 2.1 nor the 1.2 Eco R1 bands and two fragments, with molecular weights of 1.65 (Xba I origin) and 1.5 x 10^6 daltons (Eco R1 origin) are visible along with 0.5, 0.44, 0.4 and two 0.3 fragments (Track f, Fig. 15).

Digestion of the 2.1 Eco R1 band (isolated by freeze-squeeze) with Xba I gave two fragments; 1.65 and 0.40 x 10^6 daltons (Fig. 15, track c,d). Digestion of the 1.2 Eco R1 fragment gave three fragments, 0.5, 0.44 and 0.3 x 10^6 daltons (Fig. 15, track a,b). Hence it is only possible for the 0.5 Xba I fragment to be located in the centre of the 1.2 Eco R1 band (giving two Xba I sites) with the smaller fragment (0.3) adjacent to the 2nd Eco R1 site and the 0.44 fragment adjacent to the Eco R1 site 3. The third Xba I site must be just beyond the 3rd Eco R1 site to account for the other 0.5 fragment.

The final Xba I site is located in the 2.1 Eco R1 fragment, such that the small fragment, found in double digests, is adjacent to the 4th Eco R1 site. Thus, the large Xba I fragment (2.5), arises from this small fragment (0.4), the 0.3 and 1.5 Eco R1 fragments and finally the 0.3 fragment beyond the Eco R1 site 2.

Fig. 15. Electrophoretic analysis of Campbell DNA digested with Xba I restriction endonuclease (e).

(f) Campbell DNA digested with both Xba I and Eco R1.

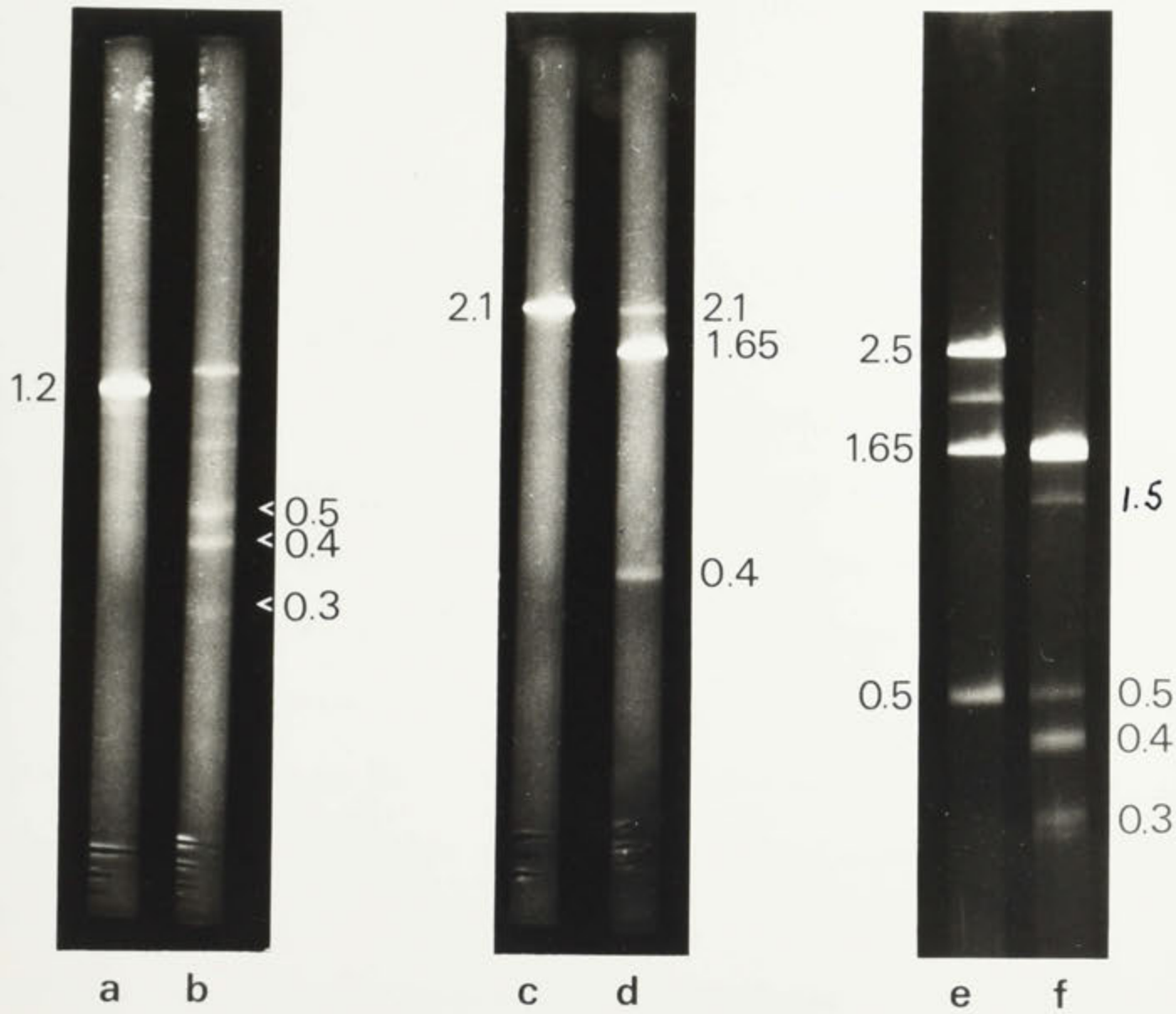
(a) Electrophoresis of the 1.2 Eco R1 fragment isolated by the freeze-squeeze method.

(b) Isolated 1.2 Eco R1 fragment further digested with Xba I. White arrows refers to bands resulting from complete digestion. Slower migrating bands are product of partial digestion.

(c) Electrophoresis of the 2.1 Eco R1 fragment isolated by the freeze-squeeze method.

(d) Isolated 2.1 Eco R1 fragment further digested with Xba I.

The top densitogram was from gel (e) and the lower from gel (f).


1.2
0.5
0.4
0.3
2.1
2.1
1.65
0.4
2.5
1.65
1.5
0.5
0.5
0.4
0.3
a
b
c
d
e
f
2.5
1.65
2.1
0.5
0.4
1.5
0.3

4.2.2.6. Hind III endonuclease

Campbell DNA digested with Hind III gave 8 definite fragments with the following molecular weights: 2.5, 0.7, 0.5, 0.37, 0.35, 0.31, 0.17 and 0.15 x 10^6 daltons (Fig. 16, track i). Also, there was always a fragment of 1.5 x 10^6 daltons present in submolar proportions. This probably was derived from linear molecules.

The molecular weights of the fragments were resolved using three different types of gels. The sizes of the larger fragments (2.5 → 0.5) were estimated in 0.8% agarose gels, the intermediate fragments (0.5 → 0.31) in 6% polyacrylamide gels and the small fragments in 10% polyacrylamide gels. A summary is presented in Fig. 16, lower half.

CaMV DNA digested with both Eco R1 and Hind III (Fig. 16, track d) gave neither 2.1 nor 1.2 Eco R1 fragments. The 1.5 Eco R1 fragment remained uncleaved. Because only one Hind III fragment, (2.55 x 10^6 daltons) is large enough to contain the 1.5 Eco R1 fragment, this provided a basis for determining the orientation of the Hind III fragments with respect to those of Eco R1.

When the 2.55 Hind III fragment was isolated (Langridge method) and subsequently digested with Eco R1, the expected 1.5 x 10^6 daltons fragment resulted. In addition fragments of 0.7 x 10^6 and 0.3 x 10^6 daltons were found (Fig. 16, track a,b). Following digestion of Campbell DNA with both Hind III and Bam I, the 2.55 Hind III fragment was cleaved and two new fragments with molecular weights of 2.[illegible] and 0.4 x 10^6 daltons appeared (Fig. 16, track c).

Considering the last two results, and the fact that the Bam I site is located in the 1.2 Eco R1 fragment, at 0.3 beyond the Eco R1 site 2, it is possible to conclude that the 2.55 Hind III fragment

runs from the 4th Eco R1 site, or very close to this point, to 0.7×10^6 daltons beyond the Eco R1 site 2 (Fig. 17).

Isolating the 2.1 Eco R1 fragment and digesting with Hind III gave four fragments of molecular weights 0.7, 0.6, 0.5 and 0.37×10^6 daltons (Fig. 16, track e,f). When digested with both Hind III and Sal I the 0.5 Hind III fragment disappeared and two fragments of molecular weights 0.28 and 0.21×10^6 daltons appeared (Fig. 16, track g,h). Since the Sal I is 0.6×10^6 daltons beyond the Eco R1 site 3, this and the two afore mentioned results indicate that the 0.5 Hind III fragment is located from 0.37 to 0.87×10^6 daltons beyond the 3rd Eco R1 site, and that one Hind III site is very close to this 3rd Eco R1 site.

This leaves the problem of locating the 0.7 and 0.6 Hind III fragments seen in the Hind III digestion of the 2.1 Eco R1 fragment. They must be between the 0.5 Hind III fragment and the 4th Eco R1 site. However, the order cannot be deduced from the available data, and hence their position relative to one another might be reversed.

The 0.6 Hind III fragment posed another problem since there was no 0.6 Hind III fragment in the Hind III digest of the native DNA. It is probable that it contained a Hind III site that was not digested when the 2.1 Eco R1 fragment was cleaved with Hind III, but that it was digested in the native sample, giving rise to the 0.31 and 0.35 Hind III fragments.

All the fragments present in the Hind III digestion have been mapped, except for the 0.15 and 0.17 fragments. Their only possible location is in the 1.2 Eco R1 fragment adjacent to the Eco R1 site 3. Since their molecular weights were very similar, it is not possible to determine their orientation with respect to one another.

Fig. 16. Electrophoretic analysis of genomic Campbell DNA digested with Hind III restriction endonuclease.

The sizes of the fragments cleaved by Hind III were assessed using three different gel systems. The molecular weight of the slower migrating bands were estimated in 1% agarose gels (i). The intermediate bands in 6% polyacrylamide gels (j) and the faster migrating bands in 10% polyacrylamide gels (k). The densitogram at the bottom corresponds to track (i).

(a) Electrophoresis of the 2.55 Hind III fragment isolated using Langridge *et al.* (in press) method.

(b) Isolated 2.55 Hind III fragment further digested with Eco Rl.

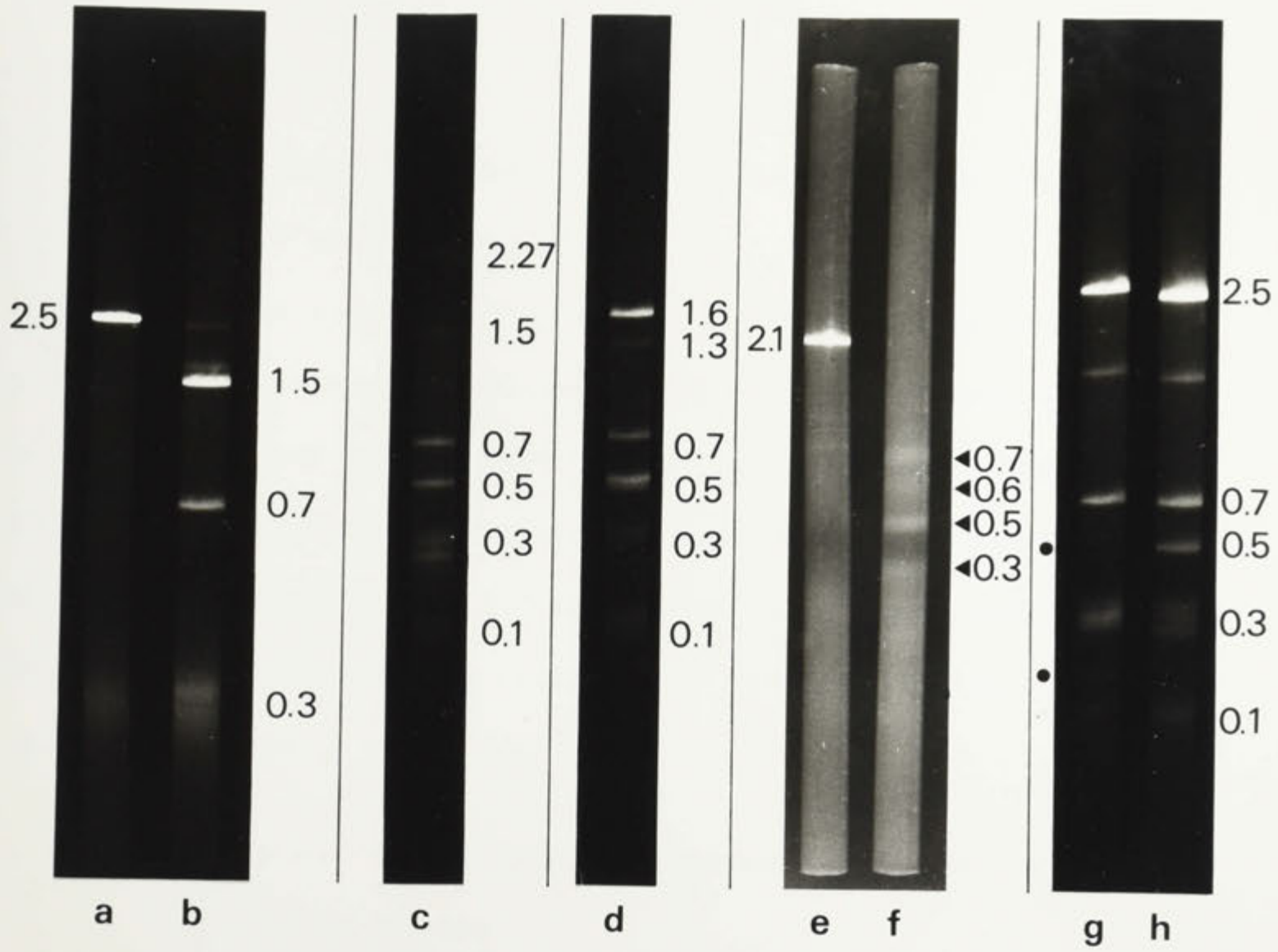
(c) Campbell DNA digested with both Hind III and Bam I.

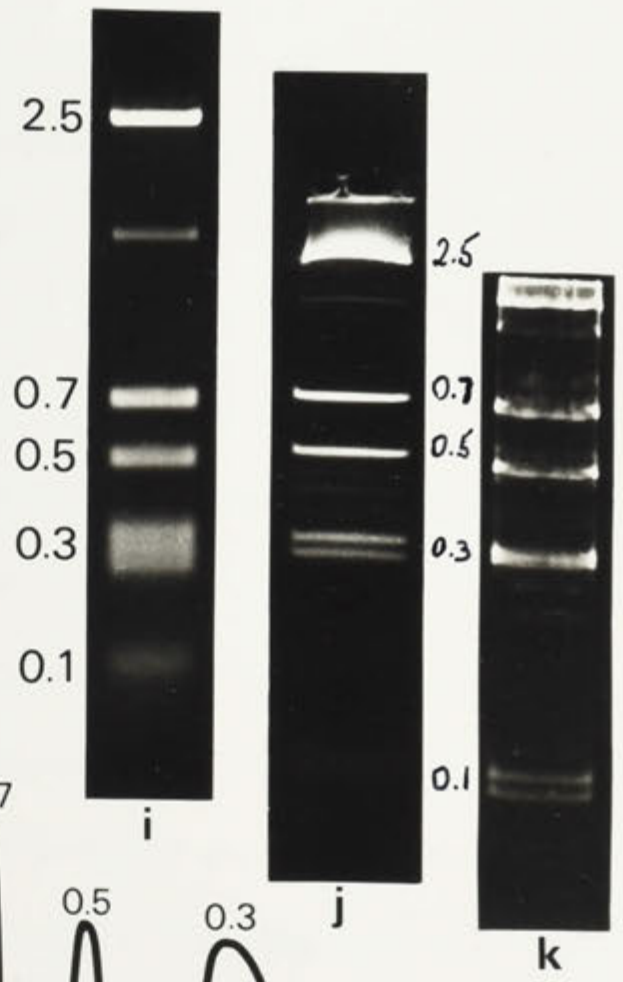
(d) Campbell DNA digested with both Hind III and Eco Rl.

(e) Electrophoresis of the 2.1 Eco Rl fragment isolated using Langridge *et al.* (in press) procedure.

(f) Isolated 2.1 Eco Rl fragment digested with Hind III. Arrows refer to the bands cleaved by Hind III.

(g) Campbell DNA digested with both Sal I and Hind III. Black dots identify missing and new bands with respect to DNA digested with Hind III alone (h).


2.5
1.5
0.7
0.3
2.27
1.5
0.7
0.5
0.3
0.1
1.6
1.3
0.7
0.5
0.3
0.1
2.1
◂0.7
◂0.6
◂0.5
◂0.3
2.5
0.7
0.5
0.3
0.1
a
b
c
d
e
f
g
h


2.5
0.7
0.5
0.3
0.1
2.5
0.7
0.5
0.3
0.1
i
j
k

2.55
1.5
0.7
0.5
0.3
0.1

4.2.2.7. Restriction endonuclease cleavage map for the genome of the Campbell isolate

When all the cleavage sites of the restriction endonuclease enzymes were determined and oriented a map for the isolate Campbell was constructed and is presented in Fig. 17. Examining this map it can be seen that numerous restriction sites are found in the region corresponding to the 1.2 Eco R1 and to a lesser extent in the 2.1 Eco R1 fragment. By contrast, no sites are present in the 1.5 Eco R1 fragment.

4.2.2.8. Restriction map for the genome of the New York and A.C.T. strains

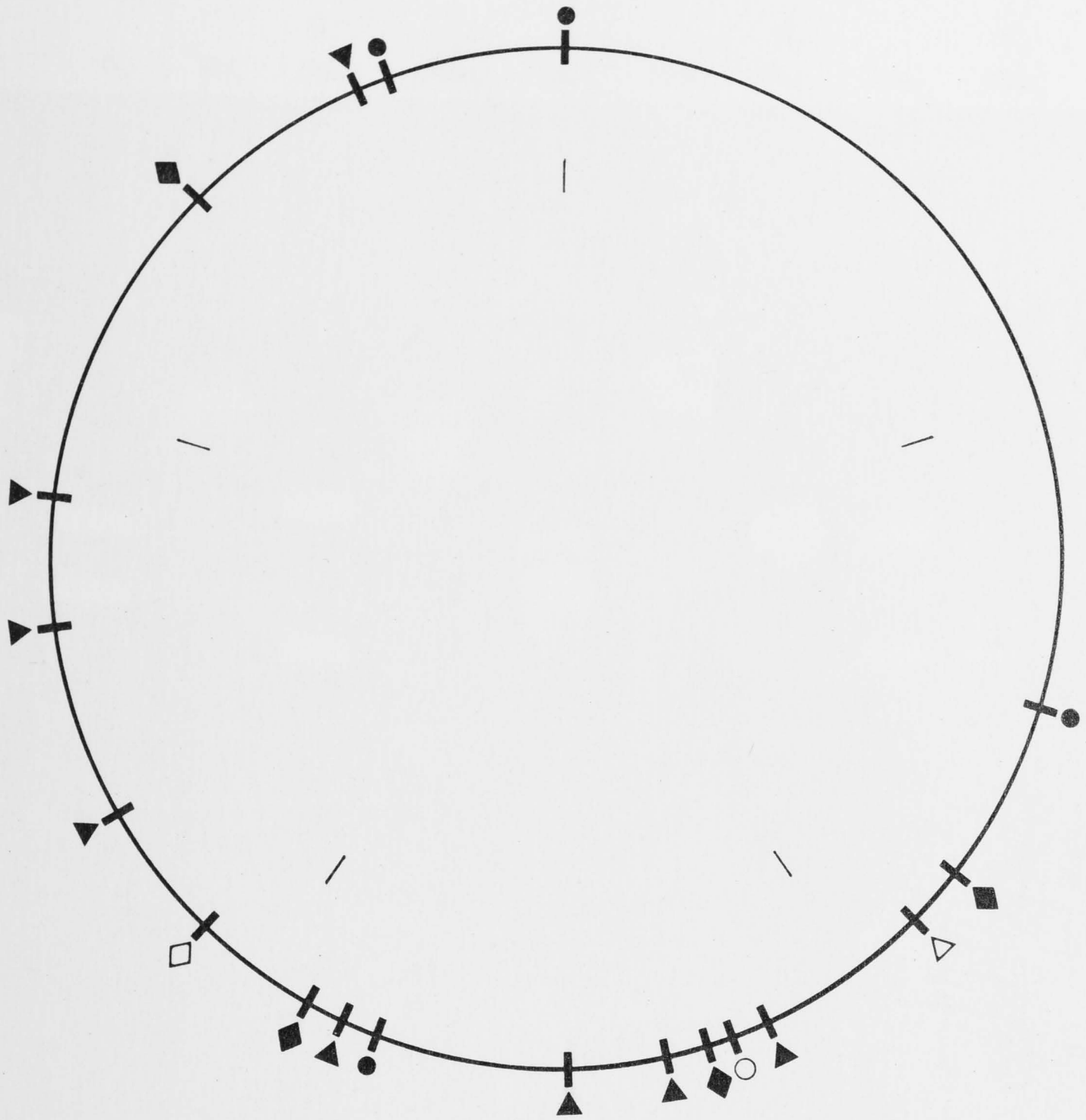
The map of these two isolates is similar to that of Campbell.

The isolate New York was more easily digested by all the enzymes than was Campbell. New York lacks the 2nd Eco R1 site present in Campbell. The three fragments obtained from the New York genome have molecular weights of 2.7 (1.5 + 1.2), 2.1 and 0.3 x 10^6 daltons (Fig. 18). The positions of all the other sites mapped were identical to those in Campbell. Both the individual and double digests with respect to Eco R1 are presented in Fig. 18.

The only other difference, which distinguishes New York from Campbell is the presence of a second Bam I site in at least part of the population, for whereas most of the New York DNA digested with Bam I produced full sized linear molecules, two bands were also present in submolar proportions with molecular weights of 3.19 and 0.2 x 10^6 daltons. The 4.8 Bam I band "might" have a second smaller band running very close to it, with a molecular weight of 4.6 x 10^6 daltons to complete the 0.2 Bam I band. The 0.2 Bam I band, as mentioned before was never visible in Bam I digests of Campbell DNA.

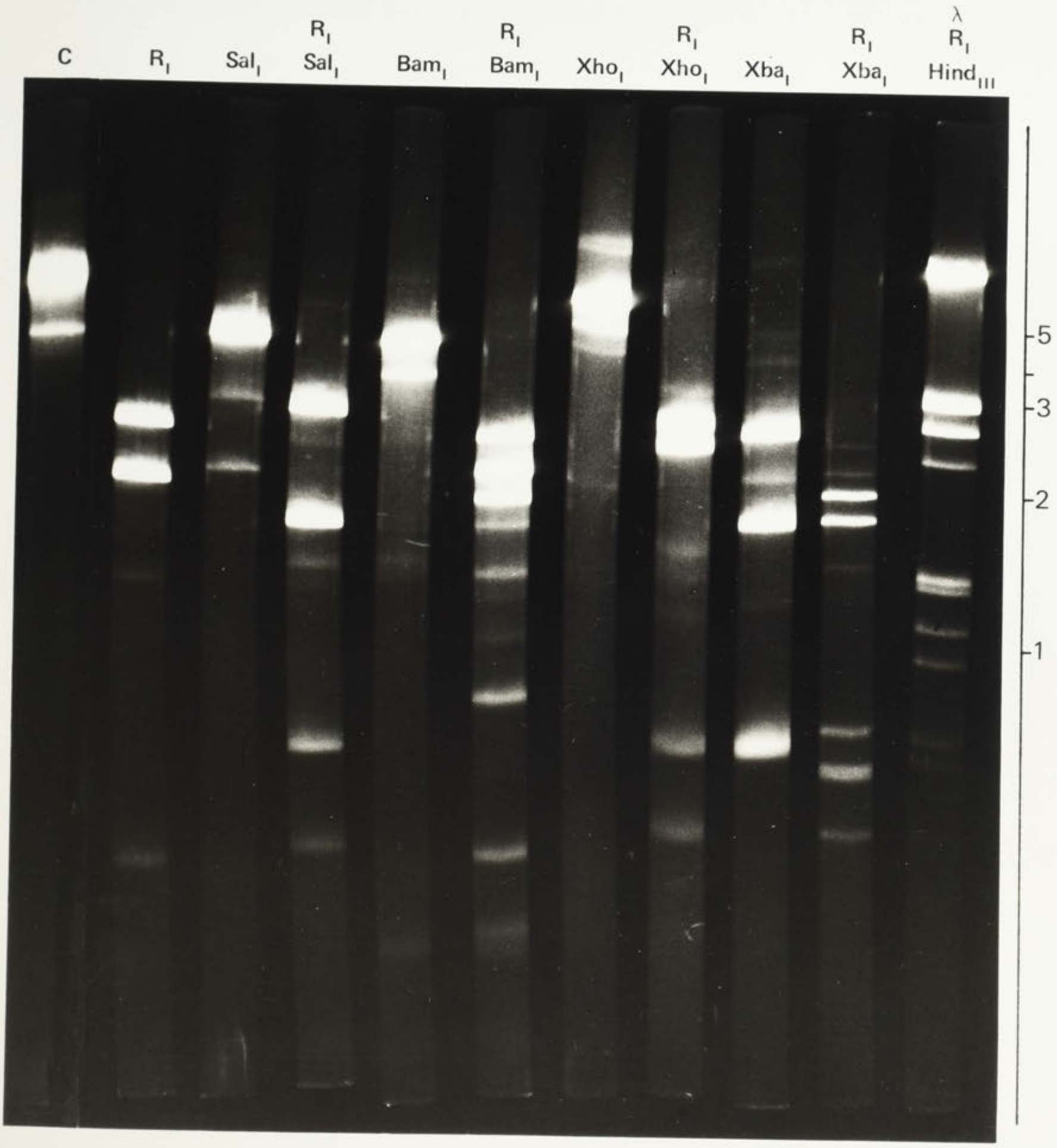
Fig. 17. Map of restriction endonuclease sites in the CaMV Campbell genomic DNA. The origin corresponds to an Eco R1 site (1) and all the other cleavage sites were oriented with respect to the sites susceptible to this enzyme. The marks (-) inside the circle represent molecular weight equivalents of 1×10^6 daltons. The total molecular weight of the genome is 5.1×10^6 daltons.

RESTRICTION MAP OF CAULIFLOWER MOSAIC VIRUS CAMPBELL GENOME



● Eco R_1
◆ Xba I
△ Bam I
◇ Sal I
○ Xho I
▲ Hind III

Fig. 18. Electrophoretic analysis of restriction fragments of the genome of the strain New York 8153 digested with Eco R1, Sal I, Bam I, Xho I, and Xba I endonucleases. Also the double digestion of each one of these enzymes and Eco R1 are presented as indicated. C refers to untreated New York DNA showing a heterogeneous population of circular (heavier) and linear (lighter) molecules. The scale on the right hand side represents molecular weights x 10^{-6} daltons.



NEW YORK 8153 DNA

The second Bam I site in New York DNA was located very close to the first site between one Xba I and the Eco R1 site 2 in Fig. 17, and was only present in a small proportion of the population.

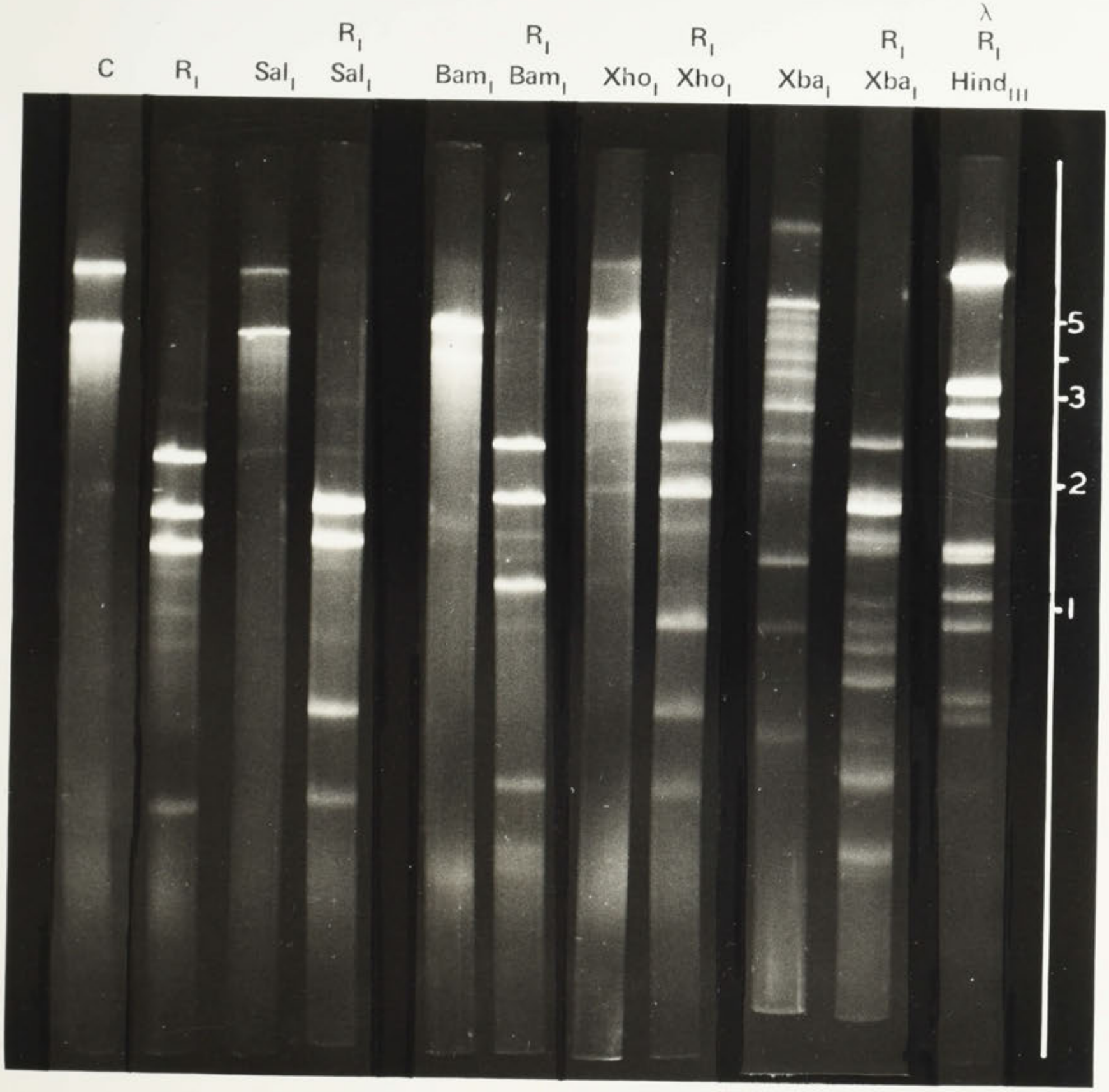
A summary of the electrophoretic analysis of restriction fragments of the A.C.T. DNA is shown in Fig. 19. The A.C.T. isolate differed from Campbell with respect to the position of two Hind III sites. A.C.T. had an extra Hind III site which mapped in the 2.5 Hind III fragment of Campbell. This resulted in additional fragments in A.C.T. of 2.1 and 0.45 x 10^6 daltons.

A.C.T. also appeared to be lacking one of the Hind III sites which was normally present in Campbell, namely that which separated the 0.37 and 0.31 Hind III fragments. This resulted in a larger fragment that coelectrophoresed with the 0.7 Hind III fragment (Fig. 20).

The A.C.T. isolate was never completely digested with Xba I under any of the conditions used, but in partially digested DNA the main bands coincided with those present in Campbell and New York, suggesting that the same sites were present.

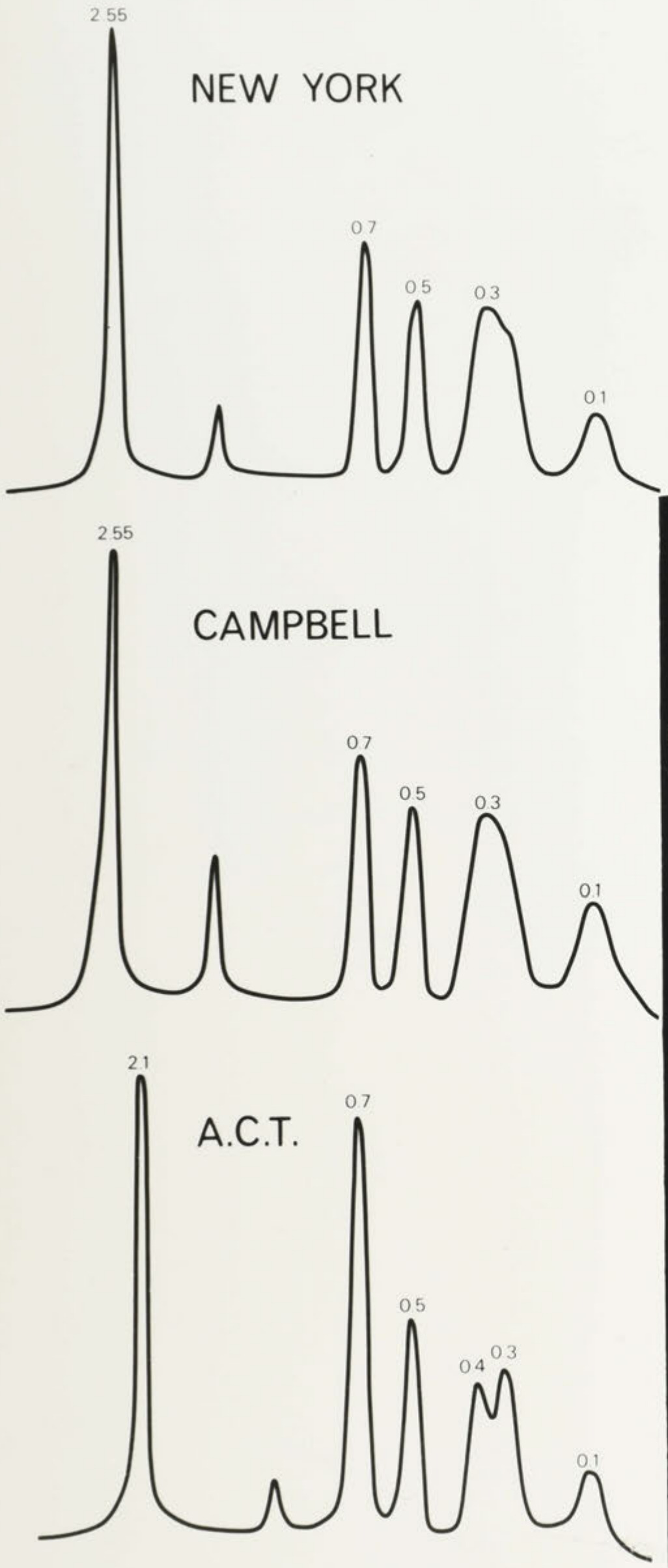
The three isolates of CaMV analysed in this study can be distinguished from each other according to their restriction endonuclease map. The enzyme Eco R1 enables New York to be separated from Campbell and A.C.T. (Fig. 21) while Hind III identifies A.C.T. as distinct from the other two (Fig. 20). These differences are presented in Fig. 22, and possible practical uses of them are discussed later in the thesis.

Fig. 19. Electrophoretic analysis of restriction fragments of the genome of the strain A.C.T. digested with Eco Rl, Sal I, Bam I, Xho I and Xba I endonucleases. Also the double digestion of each one of the mentioned enzymes and Eco Rl are presented as indicated. C refers to untreated A.C.T. DNA and the scale in right hand side represents molecular weights x 10^{-6} daltons.


C
R1
Sal1
R1 Sal1
Bam1
R1 Bam1
Xho1
R1 Xho1
Xba1
R1 Xba1
λ R1 Hind III
5
3
2
1

A.C.T. DNA

Fig. 20. Electrophoretic analysis of the New York, Campbell and A.C.T. isolates digested with Hind III restriction endonuclease. The cleavage pattern of this enzyme differentiates the strain A.C.T. from New York and Campbell.


2.55
NEW YORK
0.7
0.5
0.3
0.1
2.55
CAMPBELL
0.7
0.5
0.3
0.1
2.1
A.C.T.
0.7
0.5
0.4
0.3
0.1

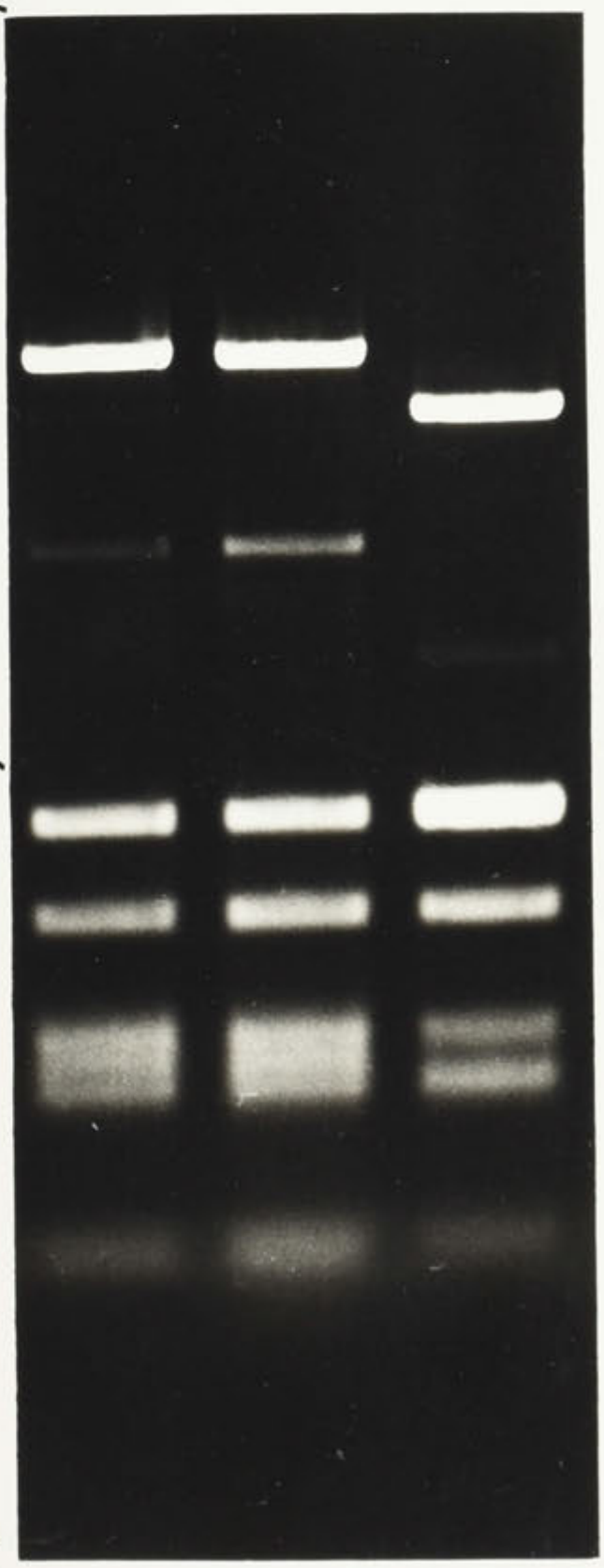
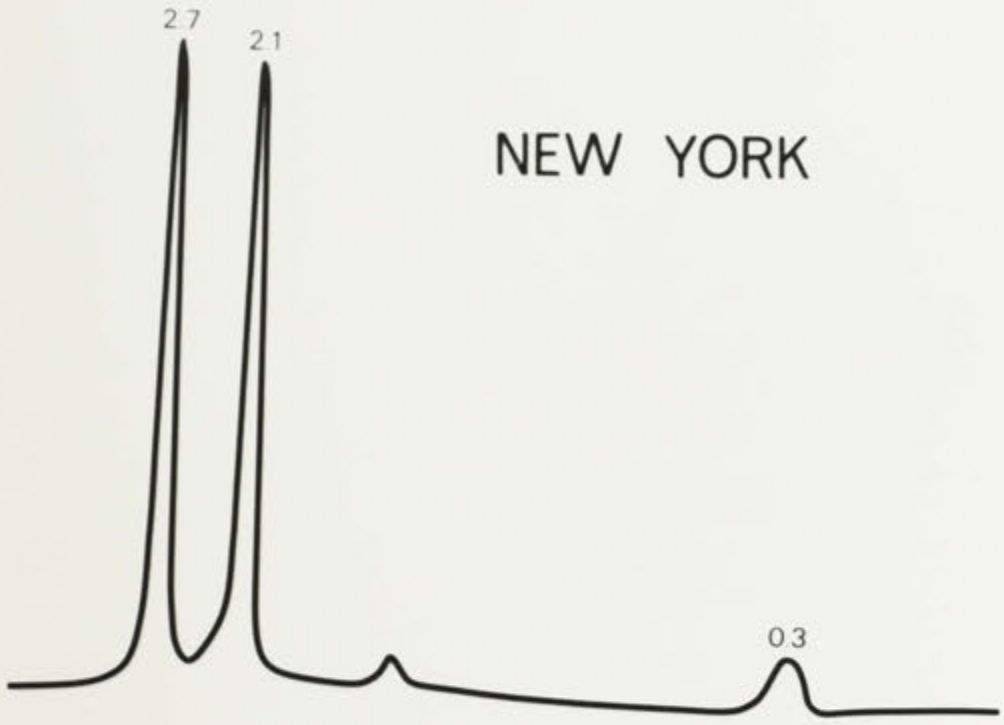
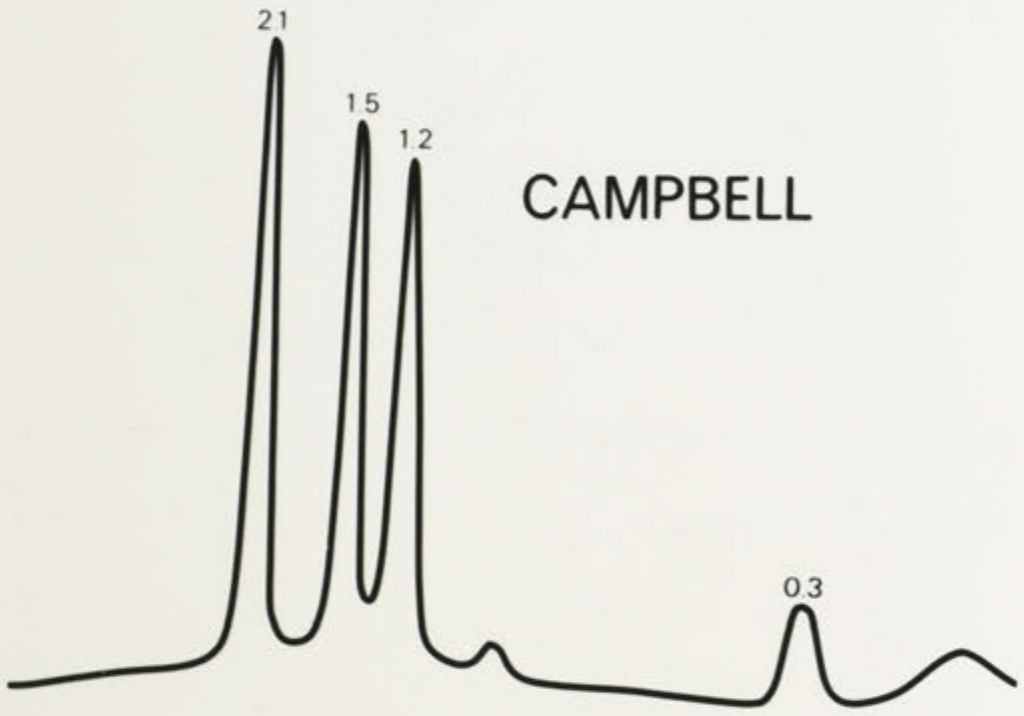

NEW YORK
CAMPBELL
A.C.T.

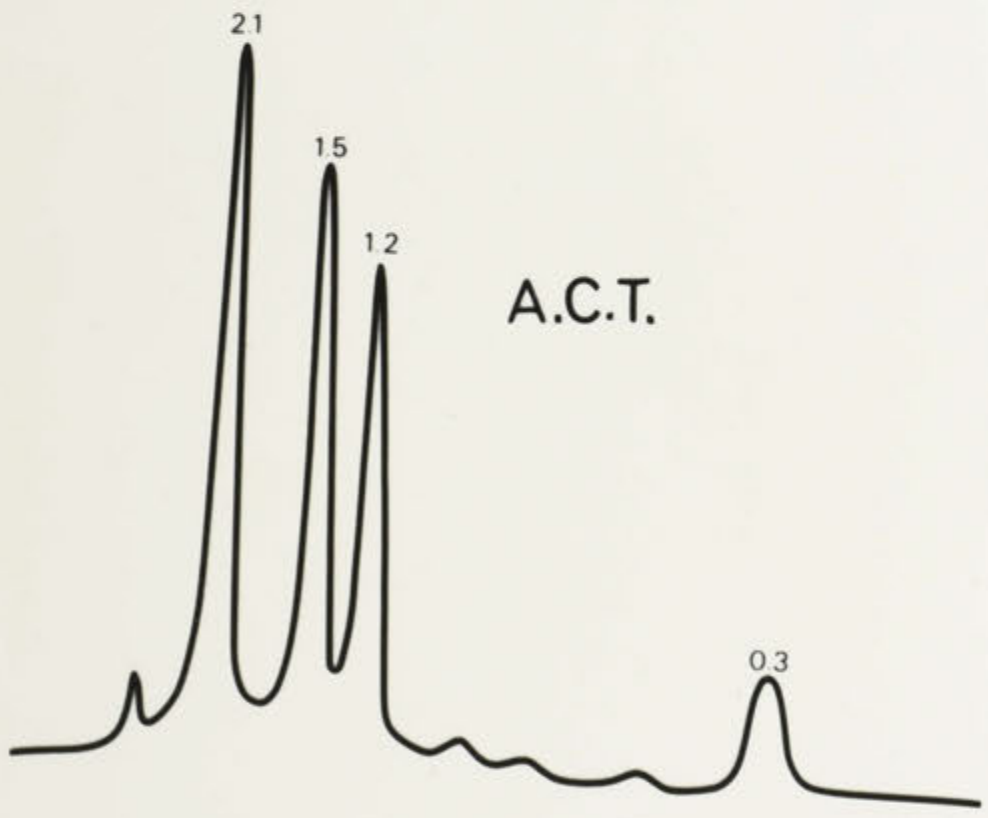
Fig. 21. Electrophoretic analysis of genomic DNA of the isolates New York, Campbell and A.C.T. digested with Eco R1 restriction endonuclease. The cleavage pattern of this enzyme differentiates the strain New York from Campbell and A.C.T.



NEW YORK



CAMPBELL



A.C.T.

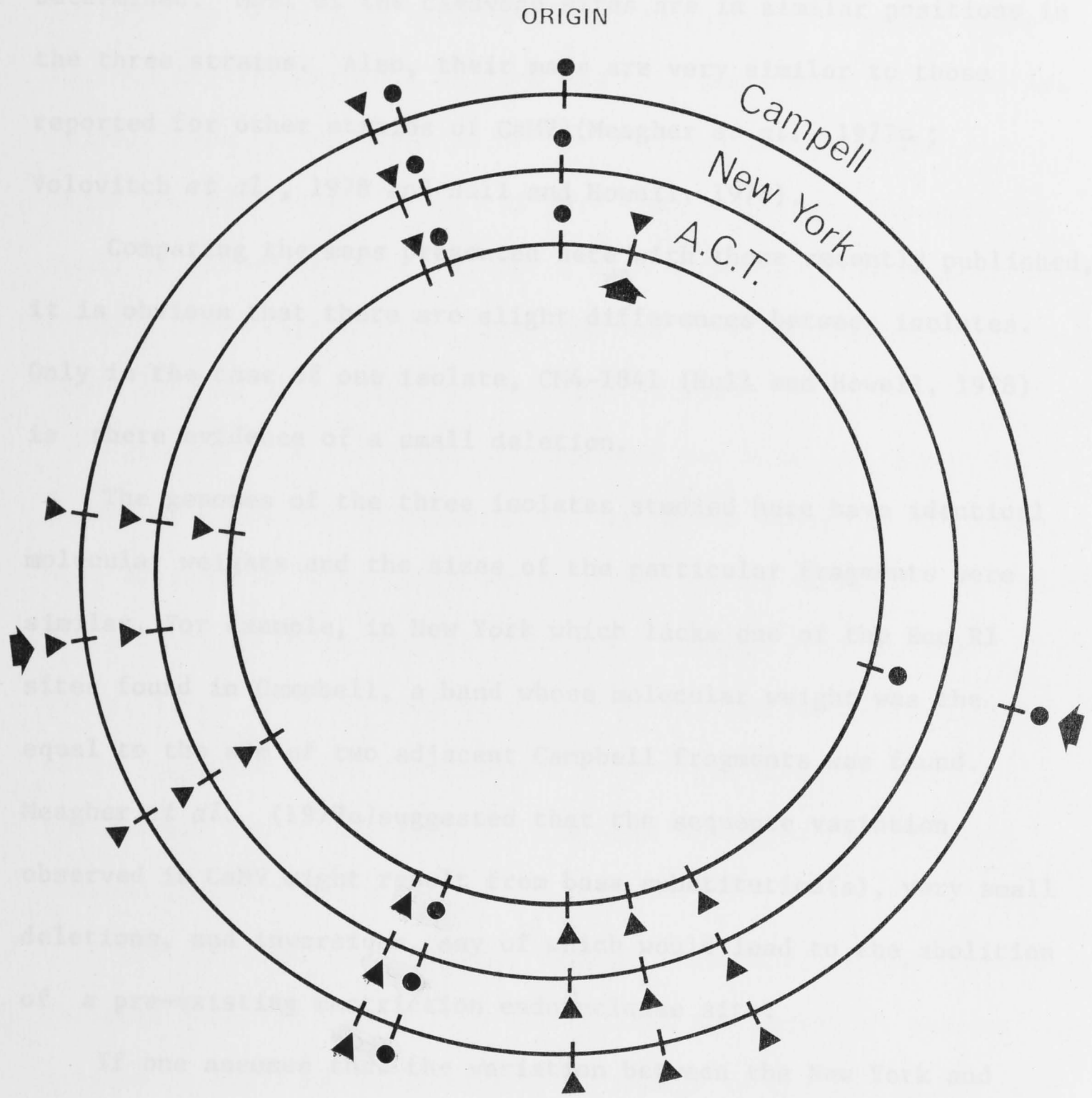
NEW YORK

CAMPBELL

A.C.T.

Fig. 22. Map of restriction endonuclease sites that allowed to differentiate the Campbell, New York and A.C.T. isolates from each other. Arrows refer to the presence or absence of sites (Eco R1 and Hind III) which distinguish the three strains.

DIFFERENCES BETWEEN THE RESTRICTION ENZYMES SITES BETWEEN CAMPBELL, NEW YORK AND A.C.T. ISOLATES



4.2.3. *Discussion*

A physical map of the cleavage sites of six restriction endonucleases in the genomes of three isolates of CaMV has been determined. Most of the cleavage sites are in similar positions in the three strains. Also, their maps are very similar to those reported for other strains of CaMV (Meagher *et al.*, 1977*a*; Volovitch *et al.*, 1978 and Hull and Howell, 1978).

Comparing the maps presented here with those recently published, it is obvious that there are slight differences between isolates. Only in the case of one isolate, CM4-1841 (Hull and Howell, 1978) is there evidence of a small deletion.

The genomes of the three isolates studied here have identical molecular weights and the sizes of the particular fragments were similar. For example, in New York which lacks one of the Eco R1 sites found in Campbell, a band whose molecular weight was the equal to the sum of two adjacent Campbell fragments was found. Meagher *et al.* (1977*a*)suggested that the sequence variation observed in CaMV might result from base substitution(s), very small deletions, and inversions, any of which would lead to the abolition of a pre-existing restriction endonuclease site.

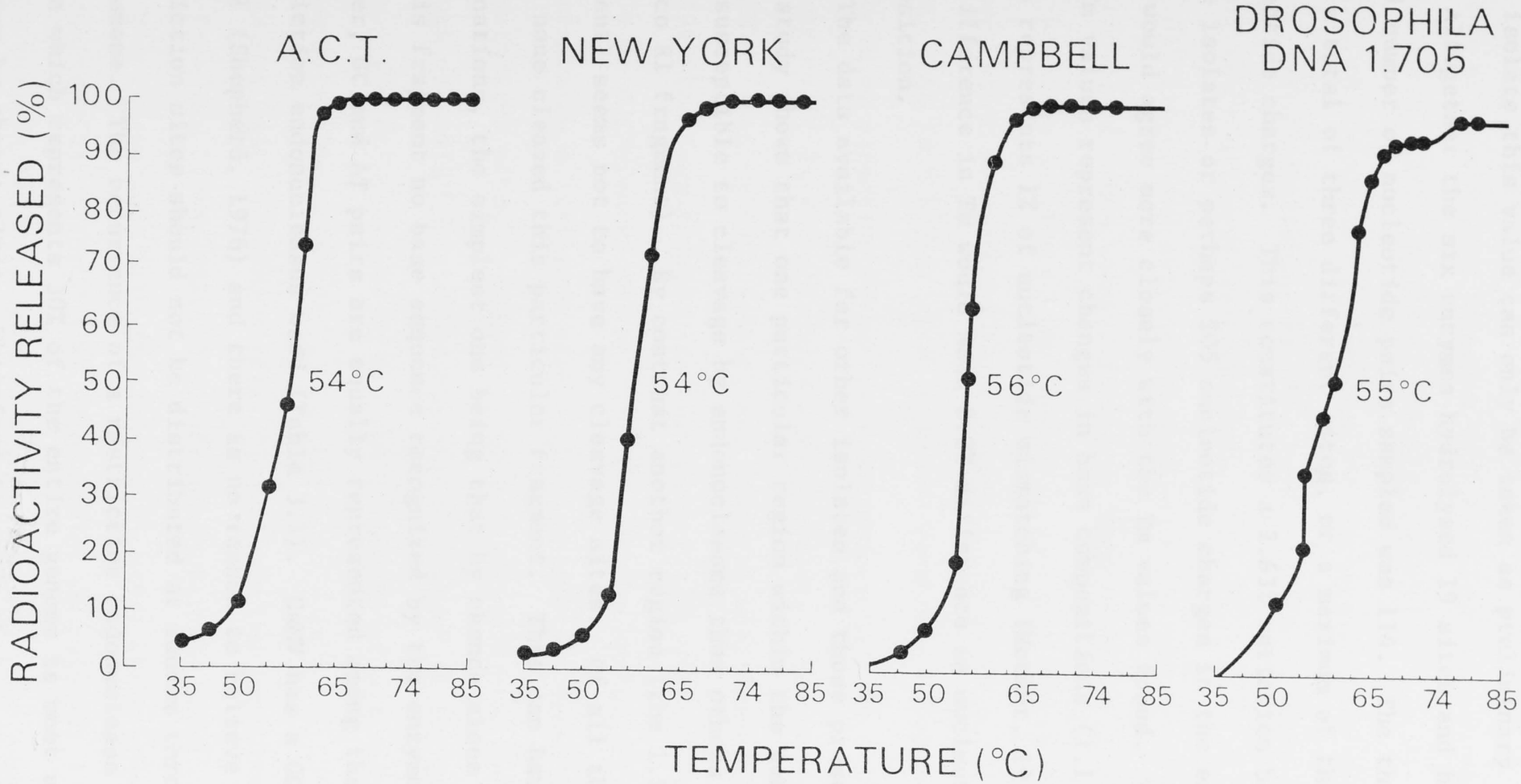
If one assumes that the variation between the New York and Campbell isolate was due to nucleotide substitutions and that differences revealed by restriction endonuclease analysis reflect the average rate of nucleotide substitution in a genome, then an estimate of sequence divergence between the strains can be obtained (Upholt, 1977; Scowcroft, 1979). This assumes that the occurrence of restriction sites is a function of the nucleotide proportions.

The molecular weight of CaMV corresponds to 7800 base pairs (Lebeurier *et al.*, 1978). The G-C content of CaMV is 43% (Schildkraut *et al.*, 1962). Based on a random distribution of nucleotides in the CaMV genome, the Eco R1 sequence, GAATTC/CTTAAG, would be expected to occur 2.39 times (Upholt, 1977). The observed number is four for the Campbell isolate and this represents 24 nucleotide pairs. Loss of one site represents a nucleotide change of at least one pair (i.e. 1/24). If this rate of change, 4%, were to apply to the whole genome it would mean a change or divergence in (1/24 x 7800) base pairs. Thus, the New York and Campbell strains could differ, in average, at 325 base pairs.

However with the other five enzymes for which no change in number of sites was encountered the estimated change is 0%. The best estimate of divergence would be the pooled estimate. Considering the nucleotide pairs sampled with the 6 enzymes used in this study, i.e., 19 sites each of 6 base pairs, the loss of one site would represent 0.87% divergence (1/(19 x 6)) between the two genomes (68.4 base pairs). The same divergence would apply between Campbell and A.C.T.

The variation in estimates between individual enzyme samples reflects expected variability when either the number of nucleotides sampled is small or the frequency of change is small. In the case of CaMV, both the number of nucleotides and the frequency, appears to be small. Even the pooled estimate of 0.87% must contain substantial uncertainty although differences in Tm between isolates do support a value of that magnitude. While determining the hybridization temperature for cRNA copies of fragments (Section 4.3), the 2.1 Eco R1 fragment was hybridized to all three strains. The Tm (Fig. 23) of the three

Fig. 23. Melting curves of the hybrids formed between ^{32}P cRNA from the 2.1 Eco R1 fragment and total A.C.T., New York and Campbell DNA. Also a melting curve for the hybrid formed between *Drosophila melanogaster* 1.705 g/cc satellite ^{3}H cRNA and unfractionated *D. melanogaster* DNA is presented. This hybrid was used as an internal standard for T_m analyses.


A.C.T.
NEW YORK
CAMPBELL
DROSOPHILA DNA 1.705
RADIOACTIVITY RELEASED (%)
100
90
80
70
60
50
40
30
20
10
0
54°C
54°C
56°C
55°C
35 50 65 74 85
TEMPERATURE (°C)

isolates was 54°C, 64°C and 56°C, but since there was only one replicate for each isolate this value can only be taken as preliminary evidence.

Altogether the six enzymes hydrolysed 19 sites and hence the total number of nucleotide pairs sampled was 114. The three strains have a total of three different sites, or a maximum of three nucleotide charges. This constitutes a 2.63% variation between the three isolates or perhaps 205 nucleotide charges in the entire genome. This would agree more closely with the Tm values found. Changes in the Tm values represent changes in base compositions (1.1°C difference in Tm represents 1% of nucleotide mismatching (Wetmur, 1976)). Thus, 2°C difference in Tm would mean 2.2% difference in nucleotide composition.

The data available for other isolates and those presented in this study shows that one particular region within the genome is more susceptible to cleavage by endonucleases than others (the 1.2 Eco R1 fragment). By contrast another region (the 1.5 Eco R1 fragment) seems not to have any cleavage sites. Of all the enzymes tried none cleaved this particular fragment. This can have several explanations, the simplest one being that by chance alone there is in this fragment no base sequence recognized by the enzymes used. However, GC and AT pairs are equally represented among the six restriction endonucleases used (Table 3.1). CaMV has a GC content of 43% (Shepherd, 1976) and there is no reason to believe that restriction sites should not be distributed at random throughout the genome. The occurrence of a restriction endonuclease resistant region which represents 30% of the entire genome is most unlikely to occur by chance alone. Therefore it seems that a special mechanism must exist to prevent endonuclease cleavage in this specific region. The most likely mechanism could be a base

modification which would render nucleotide sequences resistant to endonuclease attack. However to date attempts to demonstrate the presence of methylated bases in CaMV have been unsuccessful (Hull and Shepherd, 1977; Hull and Howell, 1978).

The main problem faced during the mapping of CaMV genome, was that of heterogeneity in the DNA of each isolate. There were clearly two sources of heterogeneity.

(a) Linear and circular molecules in the extracted DNA. This complicated the identification of bands. There is evidence that the linearization of the molecules does not result from random breakage but there appears to be a particular point at which breakage occurs. This fact became clear when the fragments obtained by endonuclease digestion of genome preparations that have different proportions of linear molecules were compared. For example, when the A.C.T. isolate (50% linear) was compared with Campbell (mainly circular) the fragmentation pattern with Eco R1 was identical, but there were differences in the intensity of the bands, reflecting differences in molar proportions. This suggests a specific "linearization point". Digestion studies using Eco R1 show that the only possible location of this site would be in the 1.5 Eco R1 fragment and would result in two new fragments of 1.2 and 0.3 x 10^6 daltons molecular weight.

In the Sal I digestion (Fig. 12), if the two submolar bands arose from the linear molecules, the linearization point would map at 0.3 x 10^6 daltons from the 2nd Eco R1 site, which coincides with the results of the Eco R1 experiments. A point of linearization at this position would also give rise to the submolar bands always present in both the Hind III and Xba I digestions. The data also indicate that the linearization point is identical in all three strains.

(b) Restriction endonuclease cleavage sites present in only a fraction of the genomes in a preparation. Such sites have been reported for other strains of CaMV (Meagher *et al.*, 1977a; Lebeurier *et al.*, 1978). In this study, preparations of all three strains showed such heterogeneity for Bam I, and in New York and Campbell for Hind III. In these examples, linearization of the molecules did not offer a satisfactory explanation for the observed heterogeneity. Bam I cut most of the DNA in one site and this linearized the molecule. This point was mapped, but in Campbell when the genome was digested with both Bam I and Eco Rl a small complementary fragment (0.3) was never found. A second Bam I site seemed to be present in a small proportion of the New York population, since a small fragment (0.2) was visible but the large complementary fragment with an expected molecular weight of 4.6 could not be distinguished from the intact linear molecule.

When Campbell and New York DNA were digested with Hind III, three fragments of similar molecular weights in the 0.3×10^6 dalton region were obtained. Two of them were always visible and the third one only occasionally. If the fragment was missing, several heavier bands, in submolar proportions could be seen (Fig. 16, track j) but a 0.3×10^6 dalton difference between the total molecular weight of fragments and the molecular weight of the genome was evident. This site, that is missing altogether in the A.C.T. strain, was eventually identified and mapped but it prevented an accurate orientation of the fragments in this region of the genome. If the site was not cleaved completely, the molecular weight of both fragments was 0.68×10^6 daltons and the adjoining

fragment had a molecular weight of 0.7 x 10^6 daltons. With the procedure followed - partial digestions - the difference in molecular weights was too small for them to be distinguished.

The map shows a discrepancy between the molecular weight of the linear CaMV DNA molecule and that derived by adding the molecular weight of the fragments. This could be due to the non-linear relationship between molecular weights and mobility in agarose gels. This is particularly applicable both to very small DNA fragments and large fragments (Danna *et al.*, 1973). The difference is always the same, when fragments are considered, as can be seen by comparing the fragments obtained by Eco R1, Xba I and Hind III digestion.

Comparing all the genomic maps available for CaMV (Meagher *et al.*, 1977a; Volovitch *et al.*, 1978; Hull and Howell, 1978; Lebeurier *et al.*, 1978; Szeto *et al.*, 1977) and those presented here it can be seen that with the exception of Szeto *et al.* (1977), the number and size of fragments obtained by each group of workers are similar. When extra sites are present they can be easily accounted for. The main difference between the reported maps appears to arise from interpretation of the orientation of the fragments with respect to the origin. For example, my results differ from those published by Meagher *et al.* (1977a) in the orientation of the Sal I site. Subsequently I used the Sal I site to locate fragments produced by other enzymes (Hind III, Bam I). So progressively, the difference between maps seems to increase. This also seems to be the case in comparing the map published by Meagher *et al.* (1977a) and Lebeurier *et al.* 1978. (Fig. 1).

Variants or mutants seem to arise within strains of the virus. Lebeurier *et al.* (1978), Volovitch *et al.* (1978) and Meagher *et al.* (1977a) have reported variants within the Cabbage B isolate. These variants might prove to be very useful in understanding the mechanism of variation in CaMV. Analysis of their genomes might show specific base substitution, inversions or recombination within the genome.

Furthermore, if the variation at the molecular level can be correlated to identifiable and measurable characteristics of the virus such as host range and infectivity, a better understanding of these phenomena can be expected.

4.3. PRESENCE OF SINGLE-STRANDED NICKS IN CaMV GENOMIC DNA, AND THEIR POSSIBLE BIOLOGICAL SIGNIFICANCE

While the restriction endonuclease fragments of CaMV were being analysed it was observed that if the DNA was heated at 60° for 5 min in the absence of salts or at low salt concentration (1 mM Tris- 0.1 mM EDTA), the molecule fragmented into at least three definite fragments. The three strains of CaMV behaved in the same way, giving fragments of the same electrophoretic mobility (Fig. 24).

The strain Campbell was selected for further experiments to determine the cause of the phenomenon. I planned to:

(a) Determine the precise temperature conditions which gave rise to the fragments and to determine if the fragments all appeared simultaneously or consecutively.

(b) Determine the nature of the fragments, to see if they were of single- or double-stranded DNA, arising from denaturation of the DNA or by breakage of the double helix.

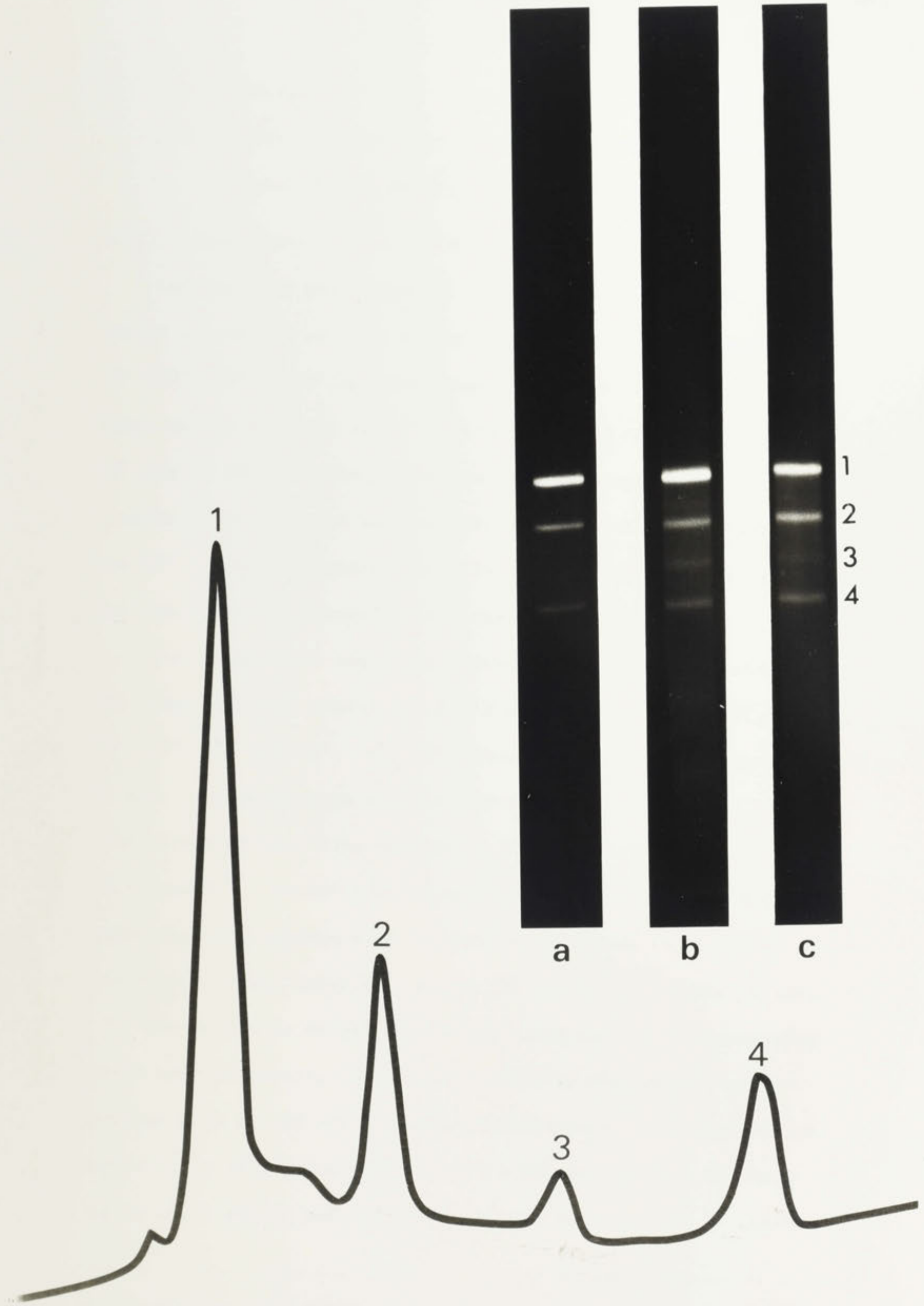
Fig. 24. Gel electrophoresis of CaMV DNA heated at 60°C for 5 min. in the absence of salts or in 1 mM Tris 0.1 mM EDTA. The gels showed 4 defined bands due to fragmentation of the DNA.

(a) New York 8135 genomic DNA

(b) Campbell genomic DNA

(c) A.C.T. genomic DNA.

The densitogram corresponds to Campbell genomic DNA (b). The number on the peaks correspond to the bands in the gel.


1
2
3
4
a
b
c
1
2
3
4

(c) Determine if there was any homology between the fragments.

(d) Locate the labile sites within the DNA molecule, with respect to the restriction endonuclease map.

4.3.1. *Conditions that cause fragmentation*

Campbell DNA was incubated in 1 mM Tris 0.1 mM EDTA, pH 8.4 for 10 min at 37° or 41°, or for 5 or 10 min at temperatures of 45°, 50°, 55° or 60° and then analysed in neutral gels. There was no breakage at 45° or less, partial breakage occurred at 50°, and at 60° the molecule was completely fragmented. Thus the fragmentation occurred when the DNA was incubated at between 45° and 55°. This experiment showed that the incubation time did not influence the fragmentation, but the temperature did.

The temperature range was further narrowed by incubating the DNA under the same conditions for 5 min at 46°, 47°, 48.5°, 50.5°, 52°, 53°, 54° and 55°. Fig. 25 shows that the first change occurred at 48°; the percentage of linear DNA molecules increased and a small fragment was first detected. Fragmentation continued at 50.5° and 52°. At 54° all the molecules had broken down into three major fragments giving rise to three clear bands (1, 2 and 4)* and one band of small molecular weight (3) present in minute amounts.

The molecular weight of the bands was assessed by comparing their mobilities with those of fragments of the phage λ genome treated with Eco R1 and Hind III endonucleases. They were found to be 1.3×10^6, 1.0×10^6 and 0.61×10^6 daltons for the major bands (1, 2 and 4) and 0.8×10^6 daltons for the band in minute

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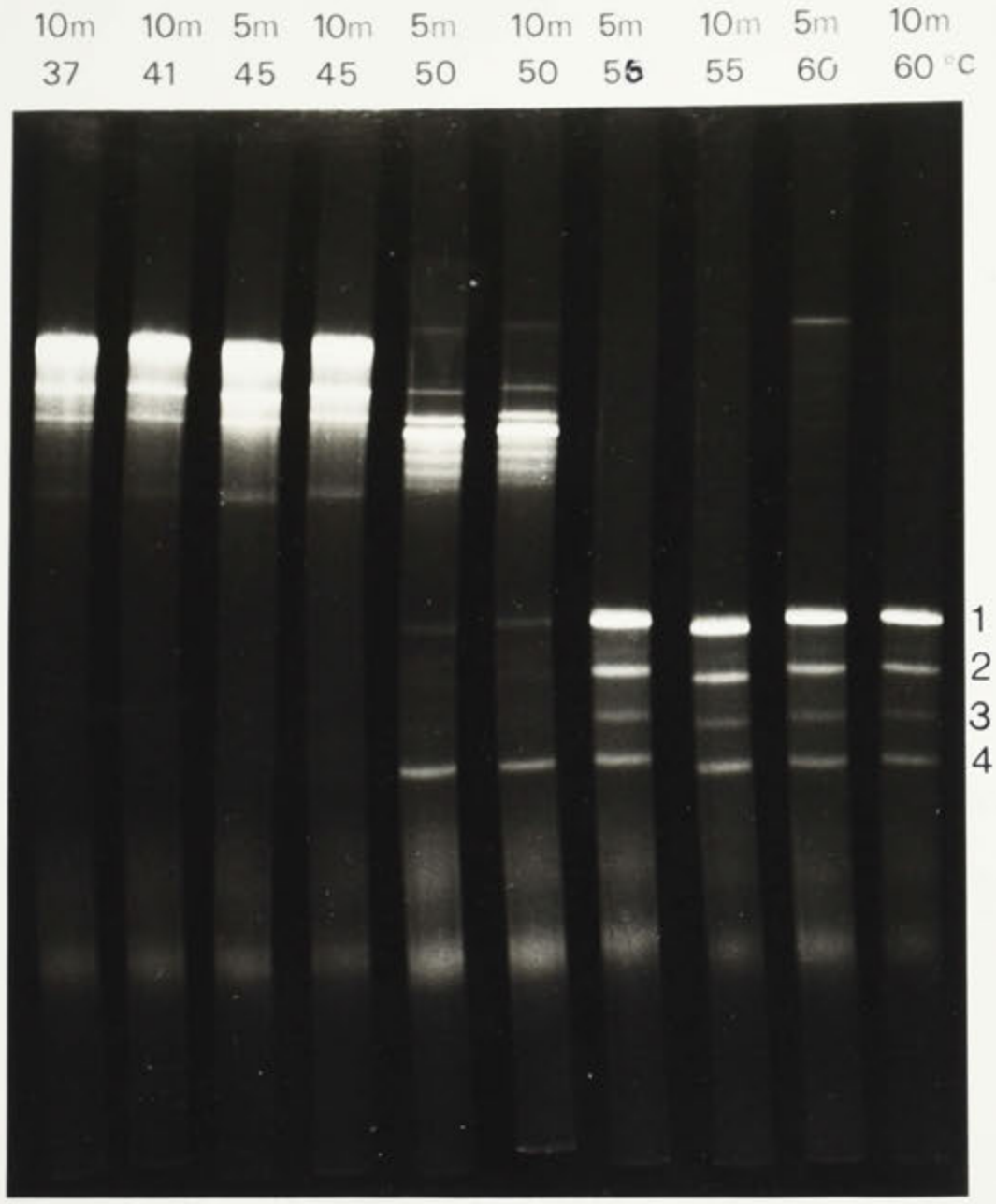
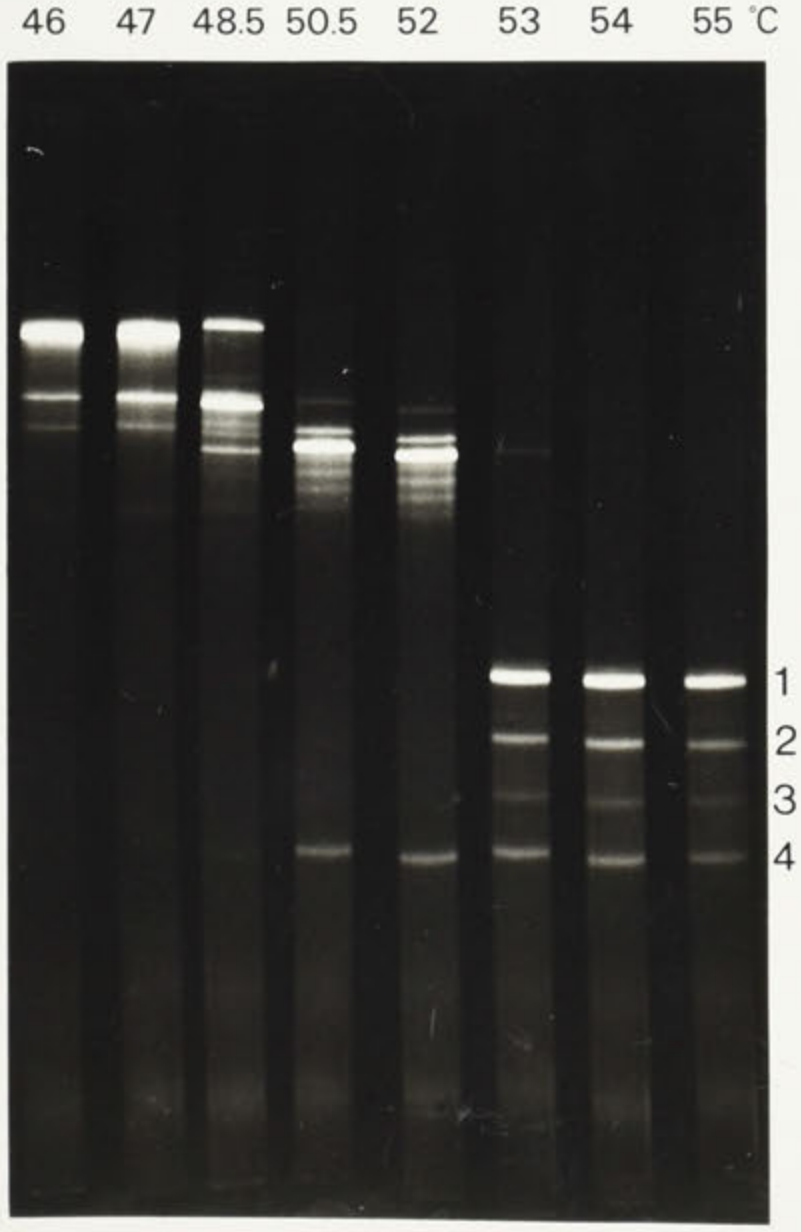
* Throughout this chapter the fragments are referred to as 1, 2, 3 and 4 in order of decreasing size.

Fig. 25. Gel electrophoresis of Campbell genomic DNA heated at different temperatures for 10 or 5 min, as indicated. The photograph at the top shows electrophoresis of DNA heated over a wide range of temperatures to determine when the fragmentation occurred. The photograph at the bottom narrows the range of temperature. In this case the DNA was heated for 5 min. only at each temperature.


10m 10m 5m 10m 5m 10m 5m 10m 5m 10m
37 41 45 45 50 50 55 55 60 60 °C
1
2
3
4


46 47 48.5 50.5 52 53 54 55 °C
1
2
3
4

amounts (3). The quantity of DNA in each of the bands was assessed by measuring its density. They seemed not to be present in molar proportions. Band 1 seemed to be in double molar proportion (Fig. 24) and band 3 was in submolar proportions but there was no other band which, together with band 3, summed to a molar quantity, and band 3 was not the result of two smaller bands missing a susceptible site. The sum of molecular weights of all the bands did not approximate the molecular weight of the intact CaMV genome. This suggested that these fragments were single-stranded rather than double-stranded.

4.3.2. *Melting curve of a CaMV DNA*

A melting curve of CaMV genome DNA in 10 mM Tris 1 mM EDTA (Fig. 26) showed a sharp denaturation curve with a T_m of 58°.

4.3.3. *Nature of the fragments generated by breakage of the molecule*

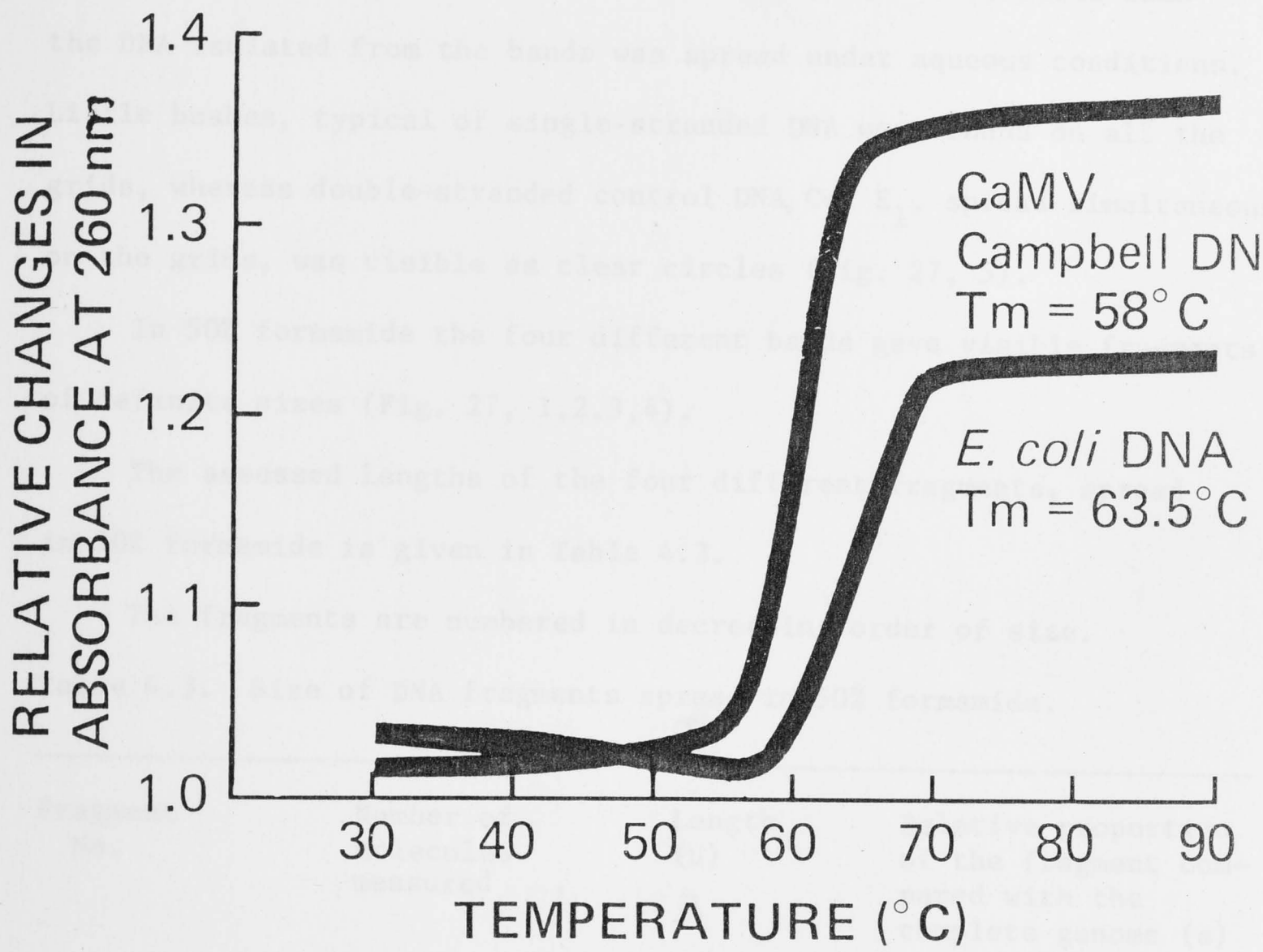
First, as discussed earlier the fragments did not occur in molar proportions. Second, the molecular weight, as determined by comparison with double-stranded fragments of the λ genome DNA seemed to be only 75% of the molecular weight of the CaMV genome. Third, there was a relation between temperature and salt concentration; higher salt concentration required higher temperature to cause the fragmentation.

All these features suggested that denaturation of the DNA was taking place and that the fragments could be single-stranded DNA. However against this hypothesis were the facts that single-stranded DNA usually does not give very sharp bands in agarose gels and that the temperature required for the fragmentation was very low.

Fig. 26. Melting curve of CaMV DNA.

The DNA was suspended in 10 mM Tris 1 mM EDTA. The changes in absorbance were measured in a Gilford spectrophotometer 2400. The temperature increased 1°C/min. using a Haake PG11 thermoregulator.

E. coli DNA was also melted under the same conditions, as an internal control.


RELATIVE CHANGES IN
ABSORBANCE AT 260 nm
1.4
1.3
1.2
1.1
1.0
30
40
50
60
70
80
90
TEMPERATURE (°C)
CaMV
Campbell DNA
Tm = 58°C
E. coli DNA
Tm = 63.5°C

4.3.4. *Electron microscopy of the fragments*

The possibility that heated DNA was denatured could be examined by electron microscopy. Under aqueous spreading conditions, if the DNA were double-stranded, linear molecules would result, whereas if the DNA were single-stranded the molecule would fold upon itself to form "bushy" structures. Under formamide spreading conditions single-stranded DNA is relaxed.

None of the fragments were visible as linear structures when the DNA isolated from the bands was spread under aqueous conditions. Little bushes, typical of single-stranded DNA were found on all the grids, whereas double-stranded control DNA, Col E_1, spread simultaneously on the grids, was visible as clear circles (Fig. 27, 5).

In 50% formamide the four different bands gave visible fragments of definite sizes (Fig. 27, 1,2,3,4).

The assessed lengths of the four different fragments, spread in 50% formamide is given in Table 4.3.

The fragments are numbered in decreasing order of size.

Table 4.3. Size of DNA fragments spread in 50% formamide.

Fragment No.	Number of molecules measured	Length (μ) [a]	Relative proportion of the fragment compared with the complete genome (a) %
1	117	2.19±0.196	94
2	71	1.42±0.308	61
3	125	0.651±0.163	28
4	151	0.535±0.092	23

(a) Length of complete genome = 2.31 μm (Shepherd and Wakeman, 1971).

Fig. 27. Electron micrograph of CaMV DNA fragments generated by breakage of the molecule. Campbell DNA was heated at 58° for 5 min. and electrophoresed in 0.8% low melting point agarose gels. The DNA fragments were isolated using the freeze-squeeze method and spread under aqueous and formamide conditions (Davis *et al.*, 1971). The grids were rotationally shadowed with platinum-paladium at low angle and examined in 1-L Phillips EM 200 electron microscope. Micrographs labelled 1,2,3 and 4 correspond to the DNA fragments 1, 2, 3 and 4, respectively, spread in 50% formamide: 5 corresponds to DNA from fragment 1 spread under aqueous conditions. The black arrow points to the little "bushes" typical of single-stranded DNA. The unfilled arrow points to a molecule of double-stranded Col E_1 plasmid DNA (molecular weight 4.2×10^6 daltons; Eshaghpour and Crothers, 1978) used as a marker.

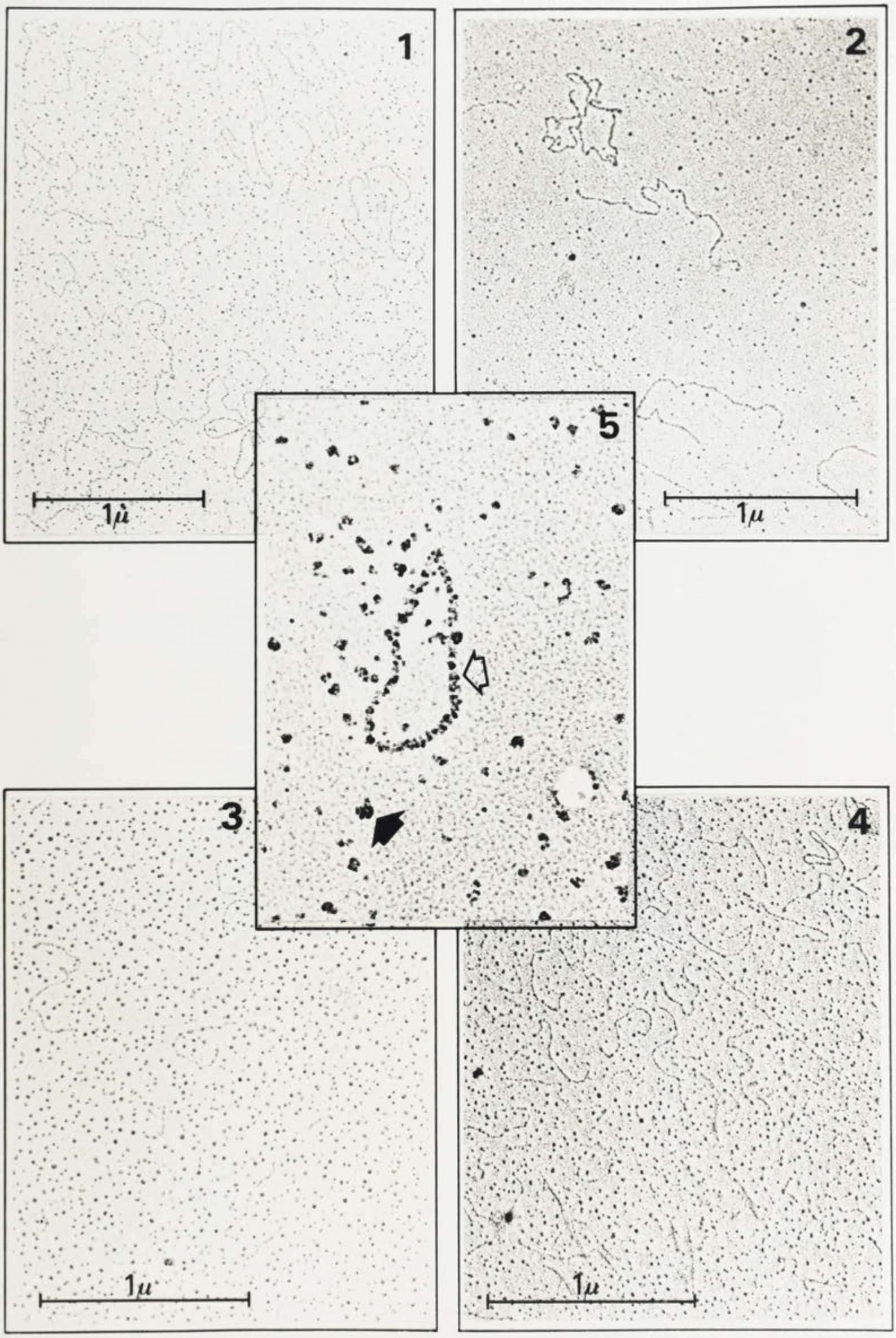
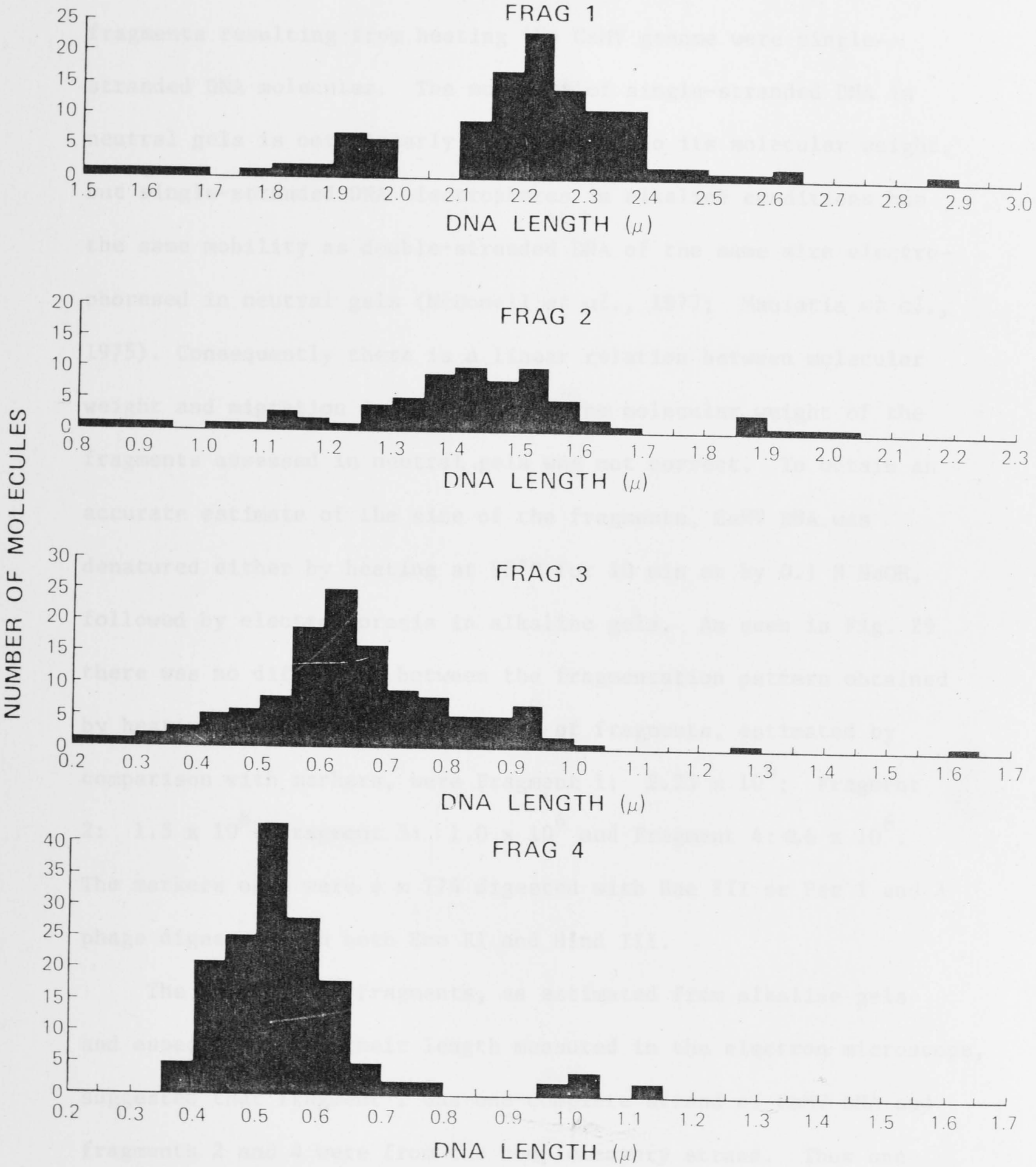

1
1μ
2
1μ
5
3
1μ
4
1μ

Fig. 28. Histogram showing the frequency distribution of the length of the DNA fragments obtained by heating. The length of the fragments was calculated from traces of a 35 mm film projected on a screen and measured with a map measurer.

DISTRIBUTION OF S-S FRAG OF CaMV



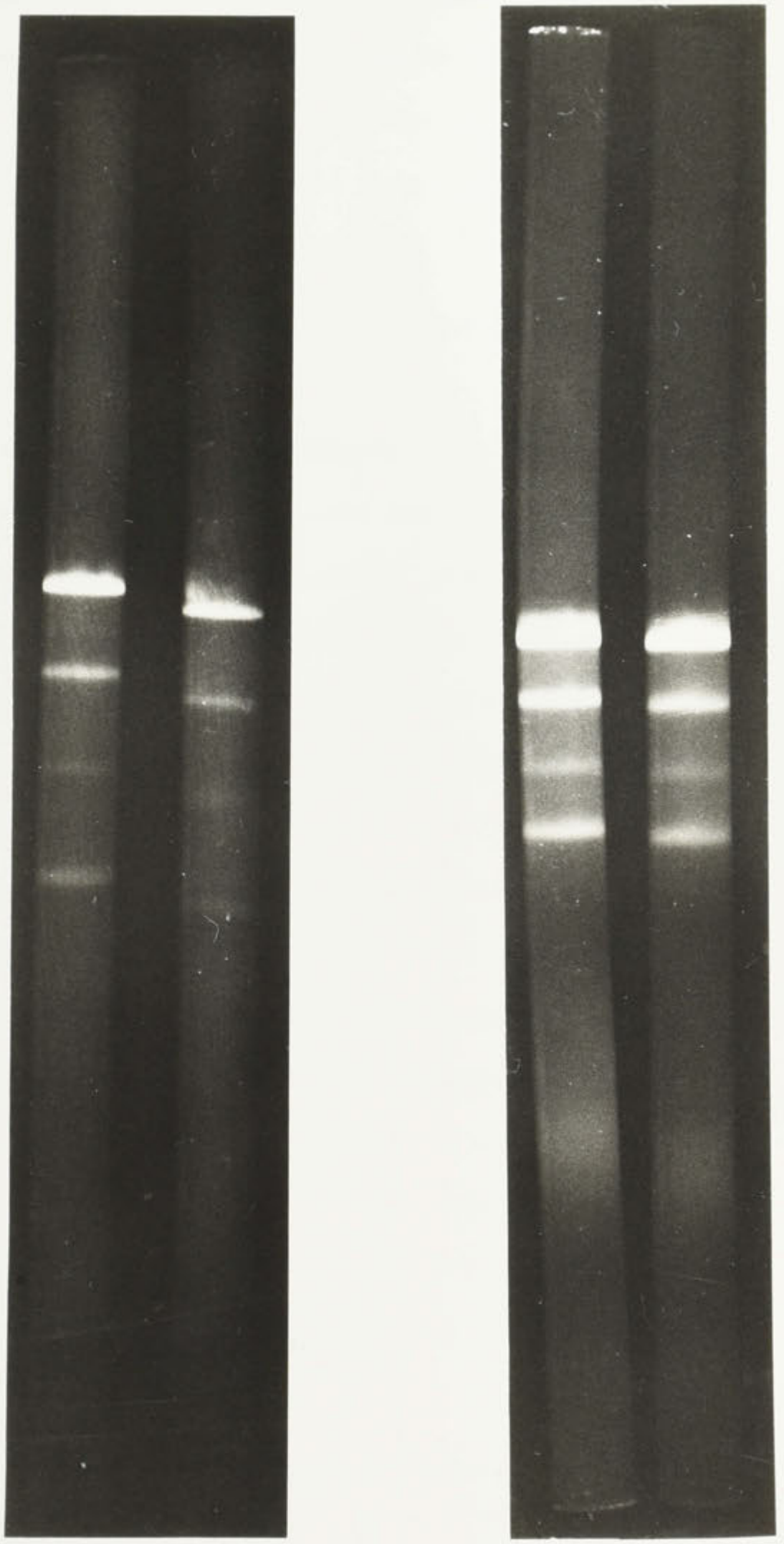
The frequency distribution for each fragment is shown as histograms in Fig. 28.

4.3.5. *Alkali denaturation of CaMV DNA*

Observations with the electron microscope indicated that the fragments resulting from heating the CaMV genome were single-stranded DNA molecules. The mobility of single-stranded DNA in neutral gels is not linearly proportional to its molecular weight, but single-stranded DNA electrophores in alkaline conditions has the same mobility as double-stranded DNA of the same size electrophoresed in neutral gels (McDonell *et al.*, 1977; Maniatis *et al.*, 1975). Consequently there is a linear relation between molecular weight and migration in gels. Hence the molecular weight of the fragments assessed in neutral gels was not correct. To obtain an accurate estimate of the size of the fragments, CaMV DNA was denatured either by heating at 98°C for 10 min or by 0.1 N NaOH, followed by electrophoresis in alkaline gels. As seen in Fig. 29 there was no difference between the fragmentation pattern obtained by heating or by alkali. The sizes of fragments, estimated by comparison with markers, were Fragment 1: 2.25×10^6; Fragment 2: 1.5×10^6; Fragment 3: 1.0×10^6 and Fragment 4: 0.6×10^6. The markers used were ϕ x 174 digested with Hae III or Pst I and λ phage digested with both Eco R1 and Hind III.

The size of the fragments, as estimated from alkaline gels and especially from their length measured in the electron microscope, suggested that fragment 1 was one complete strand of CaMV DNA and fragments 2 and 4 were from the complementary strand. Thus one strand of the genome probably has a single break, and the other has two breaks. These experiments did not suggest the origin of fragment 3.

Fig. 29. Comparison of the electrophoretic mobility of DNA fragments obtained by heating the DNA at 98°C for 10 min. (H) or by addition of alkali to 0.1 M (A). The photograph on the left hand side (alk.) shows DNA electrophoresed after treatment in alkaline gels (0.8% agarose) and the one on the right hand side (neut.) shows DNA electrophoreses after treatment in neutral agarose gels (0.8%).


alk.
neut.
H A
H A

To determine the origin of fragment 3, sequence homology between the fragments was examined. If fragment 3 originated from an extra labile site present in some proportion of the DNA population, such as fragment 1, then cRNA copies made from fragment 3 would hybridize with fragment 3 and with fragment 1.

4.3.6. *Homology of sequences between single-stranded fragments of a CaMV DNA*

The fragments were isolated from neutral gels following the method used by Langridge *et al.* (in press) and radioactively-labelled cRNA was prepared for each fragment. The cRNA copies were hybridized with denatured CaMV DNA using the technique developed by Southern (1975). As shown in Fig. 30 fragment 3 hybridized with itself, and also with fragments 1 and 2, and to a minor extent with fragment 4.

Fragment 1 hybridized with fragment 1 but also slightly with fragment 2. Fragment 2 hybridized with itself but also with fragments 1 and 3 and fragment 4 hybridized with fragments 4, 1, 2 and 3. In all comparisons, the cRNA copies hybridized not only with the homologous DNA but also to a greater or lesser extent with two or more of the other fragments. This surprising phenomenon could result from incomplete separation of the fragments during isolation or from the presence of homologous sequences within the fragments.

4.3.7. *Denaturation of the single-stranded fragments and electrophoresis*

Every experiment to confirm the purity of the isolated single-stranded fragments by electrophoresing them in neutral gels under neutral or alkaline conditions failed.

Fig. 30. Hybridisation patterns of ^{32}P-cRNA, prepared from each of the four single-stranded fragments of heat denatured CaMV, against denatured DNA immobilized on nitrocellulose filters after electrophoresis in agarose gels (Southern, 1975). Autoradiograph of filters shows pattern of ^{32}P cRNA hybridization (tracks b, c, d, e).

Upper figure

(a) Electrophoretic separation of denatured DNA in gels.

(b) Hybridization pattern of cRNA of fragment 1 to denatured DNA (a).

(c) Hybridization pattern of cRNA of fragment 2 to (a).

(d) Hybridization pattern of cRNA of fragment 3 to (a).

(e) Hybridization pattern of cRNA of fragment 4 to (a).

Autoradiograph fragments labelled a and b in track e show that fragment 1 can be resolved into two distinct fragments.

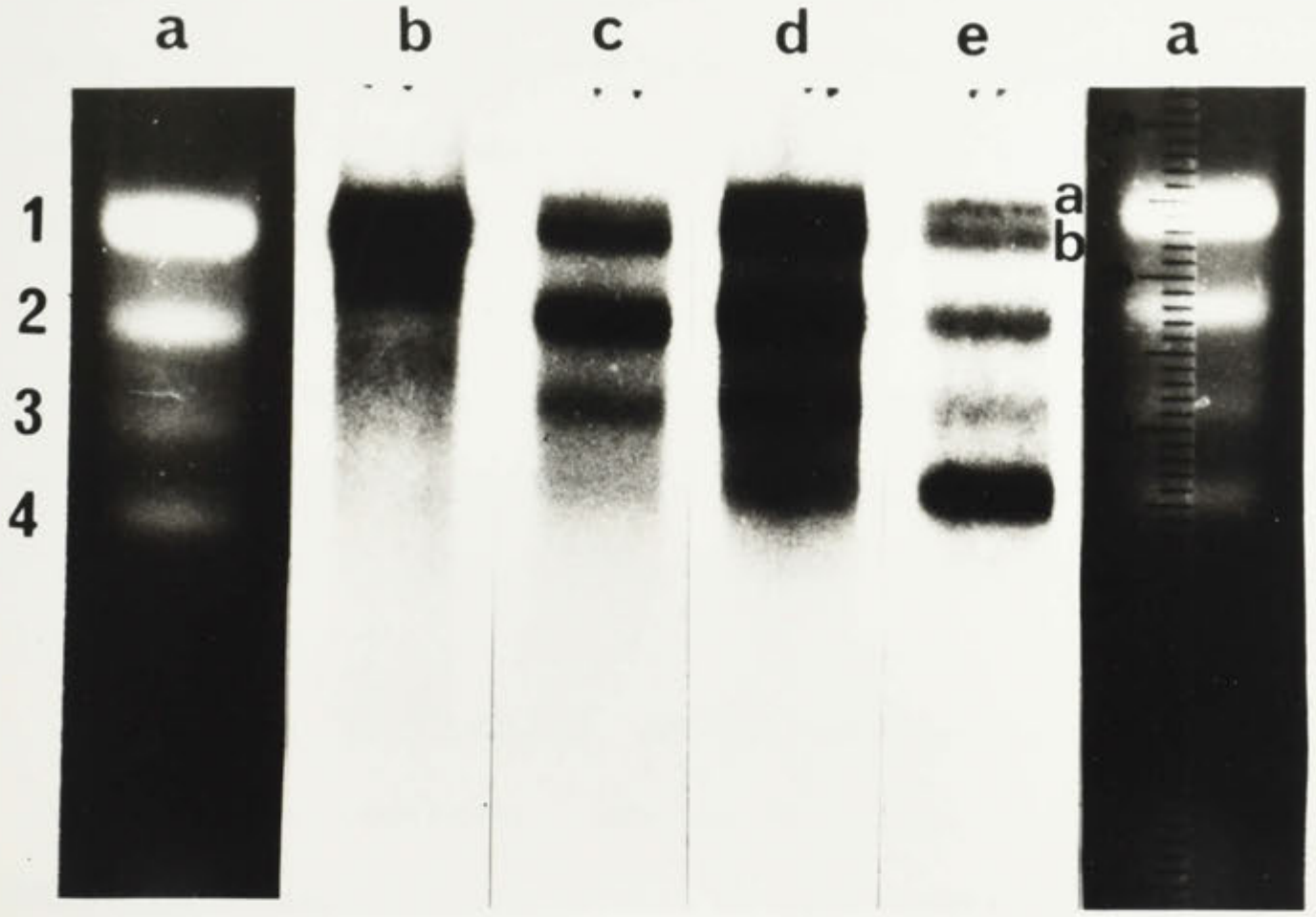
Lower figure

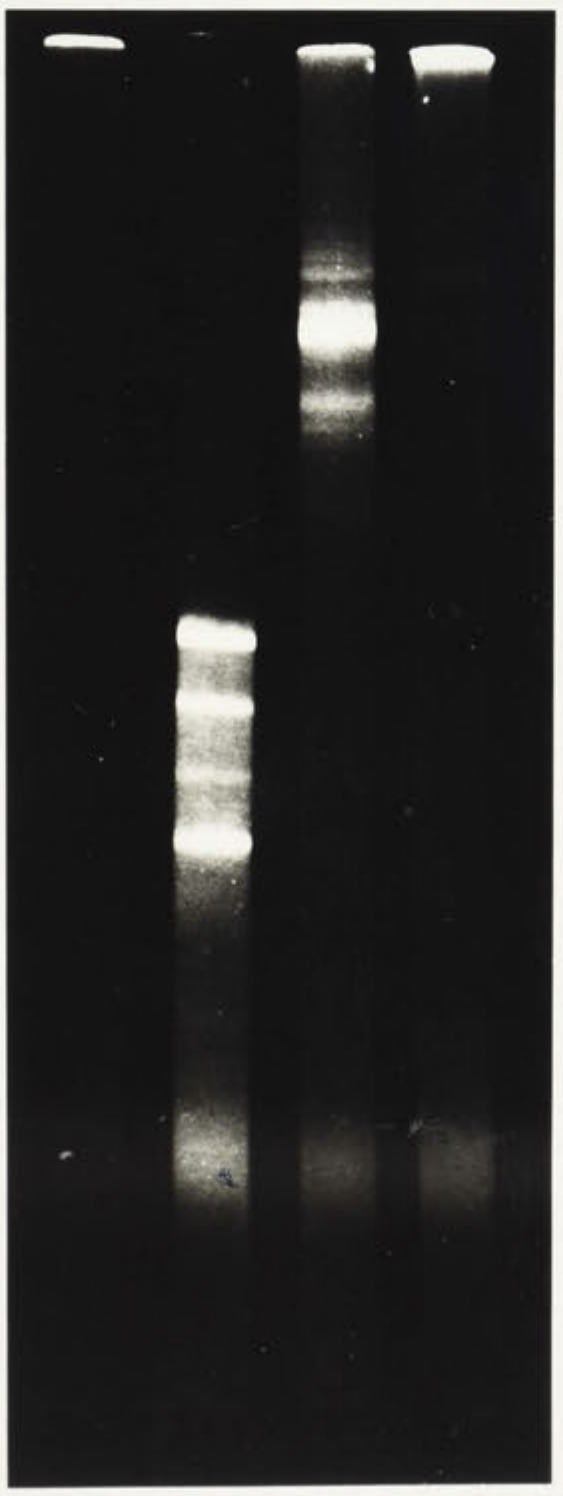
Electrophoretic analysis of single-stranded fragments isolated from 0.8% low melting point agarose. After isolation the fragments were renatured and electrophoresed again, but there was only aggregation on top of the gel (a).

(b) Denatured CaMV DNA

(c) *E. coli* plasmid pBR 322 denatured and then renatured before electrophoresis.

(d) CaMV DNA denatured and then renatured before electrophoresis. Note aggregation at top of the gel.


a
b
c
d
e
a
1
2
3
4
a
b


a
b
c
d

Each fragment was isolated using the three different procedures mentioned in Section 3.5.4, and electrophoresed separately and in different combinations with the other fragments after renaturation. All fragments aggregated at the top of the gel but did not migrate further (Fig. 30, track a).

When native CaMV DNA was denatured and renatured and subsequently electrophoresed (track d, Fig. 30) a very small proportion of molecules renatured and penetrated the gel. A large part of the DNA remained at the top of the gel and there was slight smearing along the gel.

A small *E. coli* plasmid, pBR 322, was denatured and renatured under the same conditions. As seen in Track c of Fig. 30, the DNA renatured and electrophoresed to give definite bands. Some aggregation on top of the gel and some background, as expected, was found but not to the extent found with CaMV.

The peculiar behaviour of CaMV DNA seemed to confirm the presence of homologous sequences within the genome as shown by hybridization of the single-stranded fragments. However, this did not disprove the possibility of incomplete separation of fragments. To obtain further information on the possibility of homologous sequences within the genome, restriction enzyme generated double-stranded DNA fragments of the genome were used. Double-stranded fragments are easier to isolate from gels, they can be electrophoresed again in gels to check their purity and considerably more experience is available on molecular sizing and hybridization patterns for double-stranded than for single-stranded DNA.

4.3.8. *Homology between the fragments of CaMV DNA obtained by digestion with Eco R1 endonuclease*

The four fragments derived from Eco R1 digestion of CaMV DNA

were isolated using Langridge's method (in press) and radioactively labelled cRNA copies prepared from each one of them. Each cRNA copy was hybridized against a complete Eco R1 digestion of CaMV DNA using the technique of Southern (1975). The purity of each fragment was checked by electrophoresing a fraction of each isolated DNA fragment (Fig. 31).

The T_m of RNA-DNA hybrids was 54°C for ^{32}P-cRNA made for the 2.1 Eco R1 fragment and total Campbell DNA (Fig. 23). The hybridization was done at 45° to obtain very stringent conditions. Similarly the T_m for the hybrid formed by ^{32}P-cRNA from the 1.2 Eco R1 fragment and total DNA was also 54°.

The results of the hybridization presented in Fig. 31, showed cross-hybridization between bands.

To further exclude the possibility of low levels of intraband contamination the reproducibility of this result was checked. Two different groups of turnip were grown independently in the glasshouse, and virions of CaMV Campbell purified, and then DNA isolated.

From each group a different fragment of DNA after digestion with Eco R1 was selected, fragment 2.1 from one DNA source and fragment 1.2 from the other, and the complete procedure repeated independently. The results were identical to those previously obtained.

4.3.9. *Mapping of the single-stranded nicks on the restriction endonuclease map*

To determine the location of the single-stranded nicks within the genome, the fragments cleaved by Eco R1 endonuclease were isolated using the Langridge procedure (in press), alkali denatured and electrophoresed in neutral and alkaline conditions.

Fig. 31. Hybridisation patterns of ^{32}P-cRNA, prepared from each of the four double-stranded Eco R1 generated fragments of CaMV, against an Eco R1 digest of CaMV immobilised on nitrocellulose filters following electrophoresis in agarose gels (Southern, 1975).

Upper figure

(1) Electrophoretic mobility of the Eco R1 fragments of CaMV. The numbers on the left refer to the molecular weight (x 10^{-6} daltons) of the fragments.

(2) Hybridization of ^{32}P cRNA of the 2.1 Eco R1 fragment to 1.

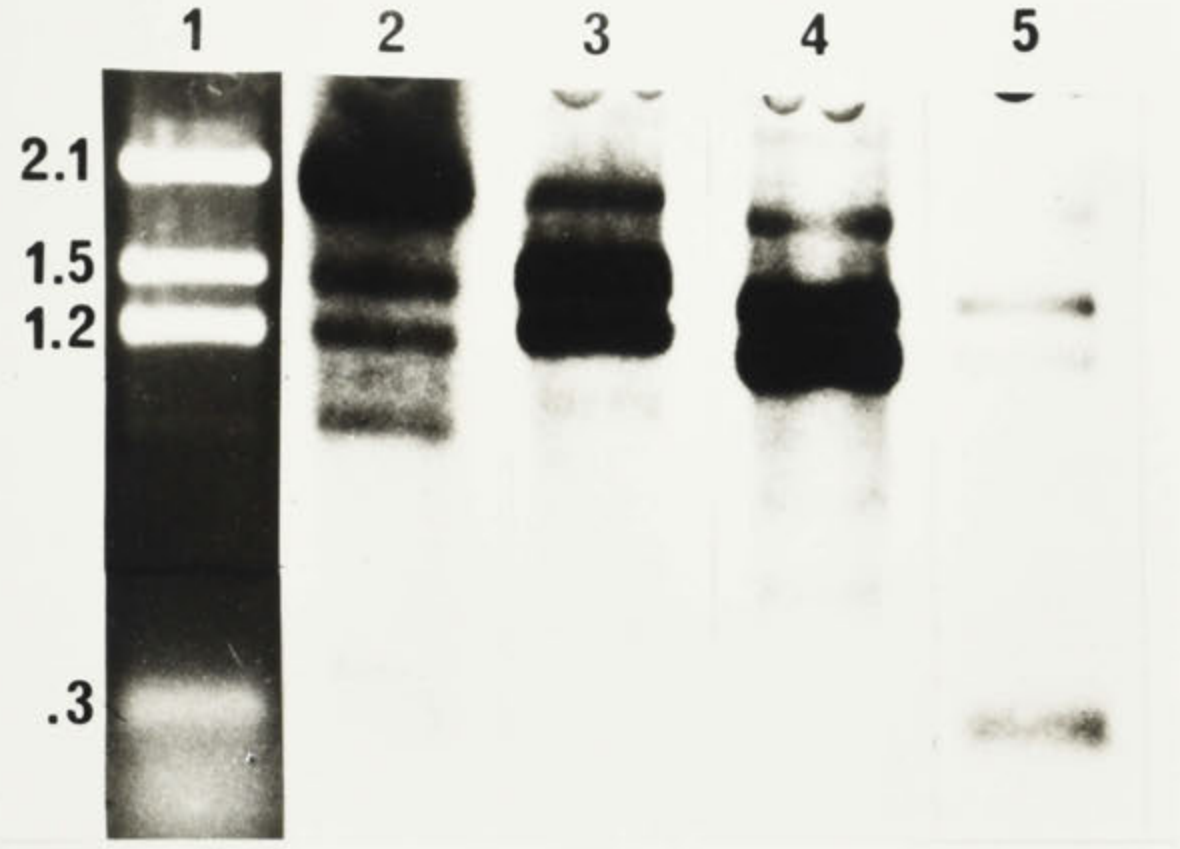
(3) Hybridization of ^{32}P cRNA of the 1.5 Eco R1 fragment to 1.

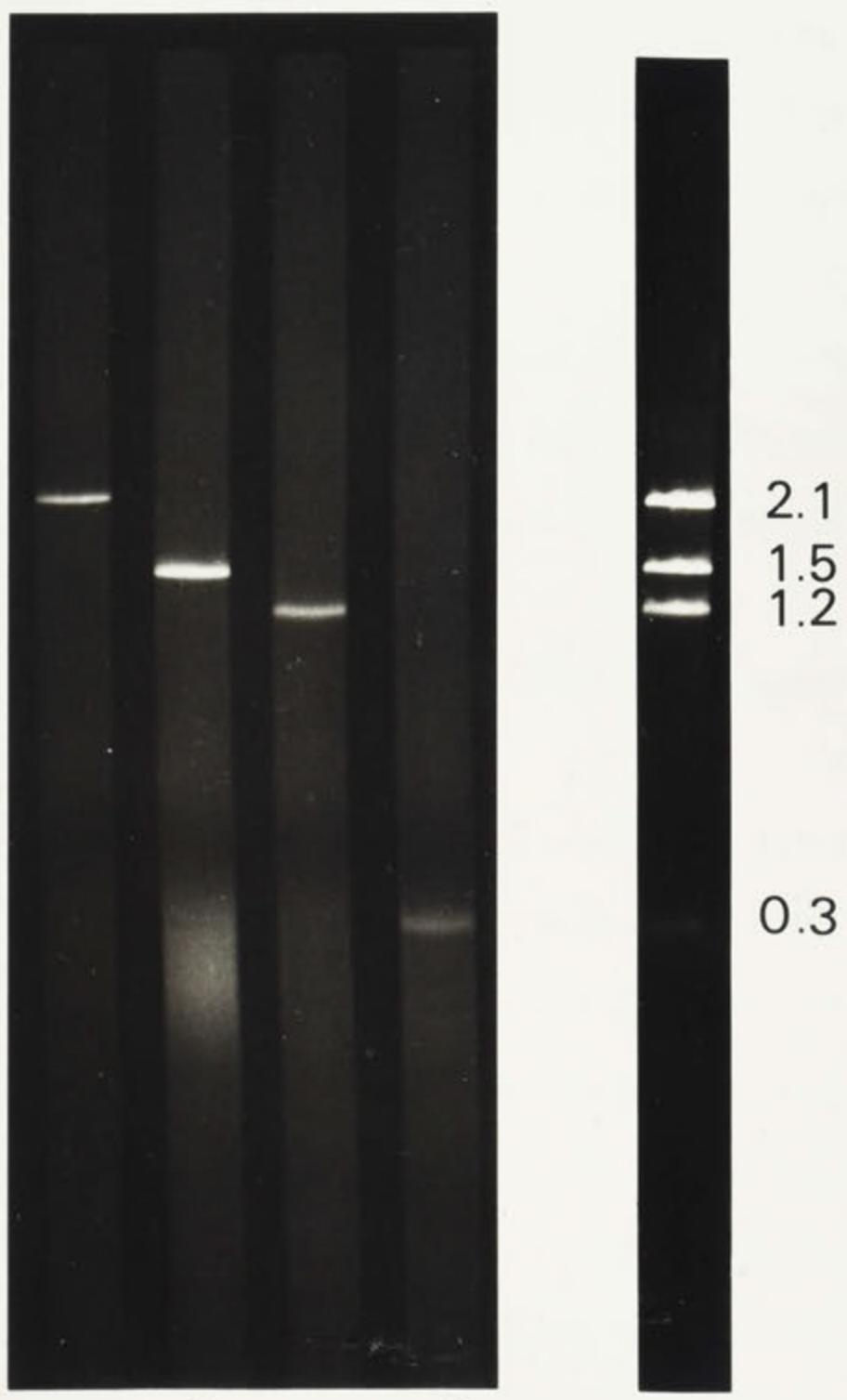
(4) Hybridization of ^{32}P cRNA of the 1.2 Eco R1 fragment to 1.

(5) Hybridization of ^{32}P cRNA of the 0.3 Eco R1 fragment to 1.

Lower figure

Electrophoresis of each of the four Eco R1 fragments after isolation from agarose gels using Langridge method (in press). (a) 2.1 fragment; (b) 1.5 fragment; (c) 1.2 fragment; (d) 0.3 fragment. There was no indication of intra-band contamination. (e) Shows CaMV DNA after digestion with Eco R1 restriction endonuclease.


1
2
3
4
5
2.1
1.5
1.2
.3


2.1
1.5
1.2
0.3
a
b
c
d
e

The results in Fig. 32, Track b show clearly that one break is located in the 2.1 Eco R1 band. Upon denaturation this fragment gives 3 bands which according to their mobility in alkaline gels (Fig. 32, 1b) have molecular weights of 1×10^6, 0.6×10^6 and 0.4×10^6 daltons.

The 1×10^6 band corresponds to one complete strand and the other two to the complementary strand.

The 1.5 Eco R1 band upon denaturation gave two bands with a molecular weight of 0.75×10^6 and 0.7×10^6 (Fig. 32, track d, 1d). The 0.75×10^6 band corresponds to a complete strand but the 0.7×10^6 one could correspond to the complementary strand which has lost a small fragment due to the presence of a single stranded nick.

The 1.2 Eco R1 band gave one distinct band which corresponded to an intact single-stranded fragment (0.6×10^6 daltons) and a very faint band of a molecular weight of 0.5×10^6 dalton (Fig. 32, track f, 1f).

The 0.3 Eco R1 band gave only one band with the original molecular weight (Fig. 32, track h, 1h).

Thus it appears that two single-stranded nicks are relatively close to the Eco R1 restriction sites. But there is also the possibility that the two bands obtained from the 1.5 and 1.2 result from the separation of two complete single-strands. Hayward (1972), using similar conditions to those used in this experiment, demonstrated that in some phages, such as λ, T_2 H, T_1 and T_7, the denatured DNA genome gave two bands representing the separated DNA strands. But, he also found that other phages such as P_1 did not exhibit this characteristic giving only one band.

The enzyme, Xba I, hydrolyses four restriction sites in CaMV DNA,

Fig. 32. Electrophoretic analysis of denatured Eco R1 fragments. The Eco R1 fragments were isolated (Langridge, in press), denatured and electrophoresed in neutral (top picture) and alkaline agarose gels (bottom picture).

(a, 1a) Campbell1 DNA digested with Eco R1.

(b, 1b) 2.1 Eco R1 fragment after denaturation

(c) 2.1 Eco R1 fragment.

(d, 1d) 1.5 Eco R1 fragment after denaturation

(e) 1.5 Eco R1 fragment.

(f, 1f) 1.2 Eco R1 fragment after denaturation.

(g) 1.2 Eco R1 fragment.

(h, 1h) 0.3 Eco R1 fragment after denaturation.

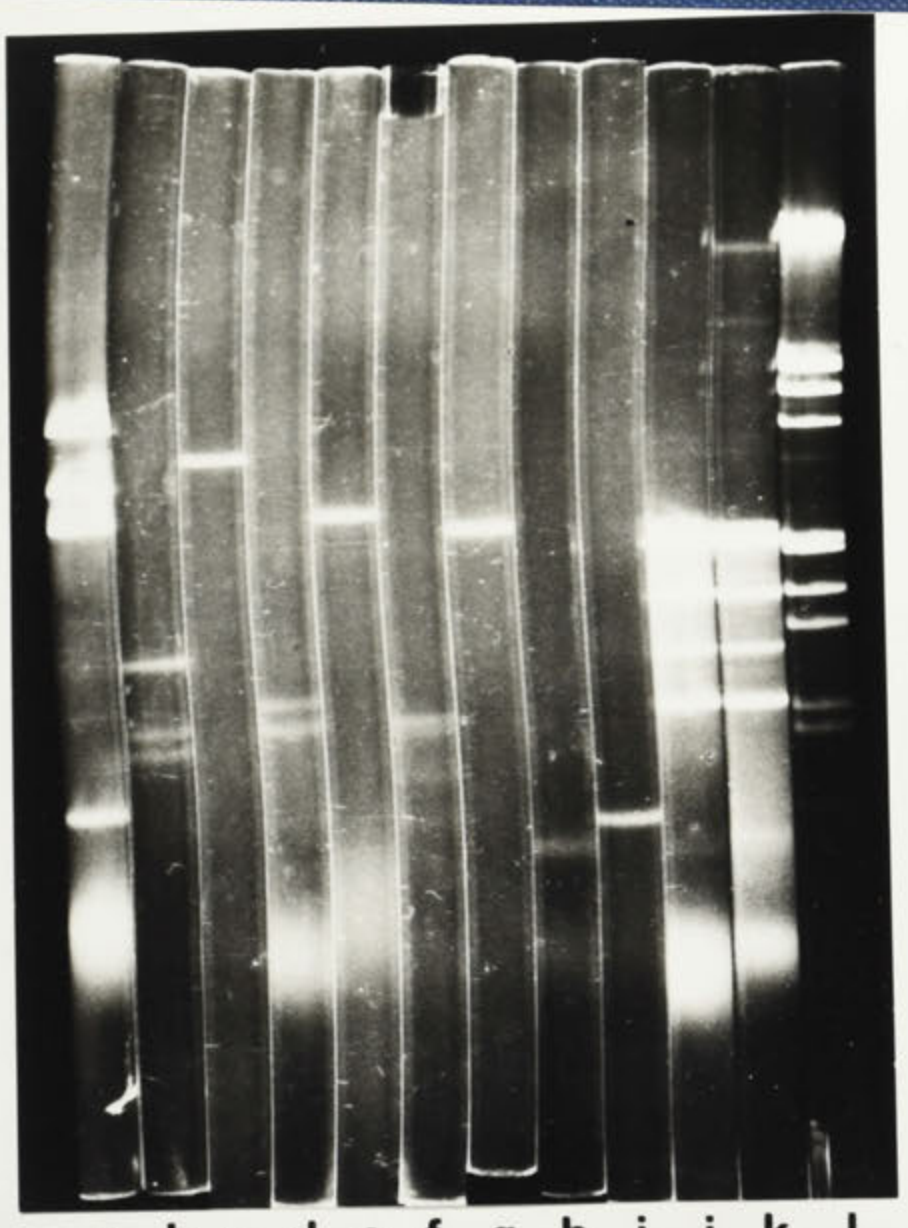
(i) 0.3 Eco R1 fragment.

(j, 1j) Campbell1 DNA denatured by addition of alkali.

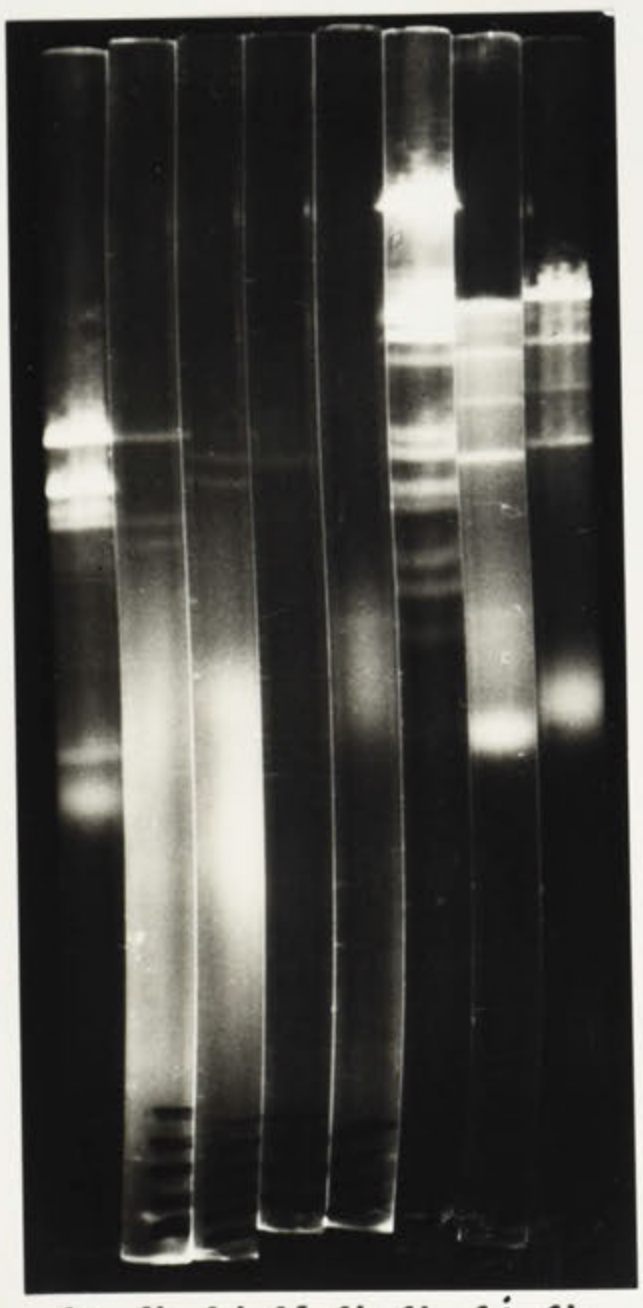
(k, 1k) Campbell1 DNA denatured by heating at 98°C for 10 min.

(1, 11) λ DNA digested with both Eco R1 and Hind III.

The sizes of the fragments are presented in Fig. 34 together with their orientation.



a b c d e f g h i j k l



1a 1b 1d 1f 1h 1l 1j 1k

and these are relatively distant from the Eco R1 sites and hence can be used to verify the presence of the gaps in the 1.5 and 1.2 Eco R1 fragments. If the 1.5 Eco R1 fragment did have a single stranded nick close to one end, the 2.5 Xba I fragments produced by cleavage of CaMV DNA by Xba I (see restriction map for the Campbell isolate Fig. 17) should give three relatively large bands upon denaturation.

If the third gap is closed to the Eco R_1 site 3, one of the 0.5 Xba I fragments upon denaturation should give rise to a second band. Furthermore the intensity of fluorescence of the bands should be quite different reflecting their different molarities.

The 1.65 Xba I fragment should also be fragmented due to the gap already mapped in the 2.1 Eco R_1 fragment.

4.3.9.1. Denaturation of the Xba I fragments.

The Xba I fragments were isolated by the Langridge method (in press), denaturated by addition of alkali and electrophoresed in neutral gels (Fig. 33).

The 2.5 Xba I fragments gave four well defined bands verifying the presence of a nick near the end of the 1.5 Eco R1 fragment and the possible linearization of the molecule.

The 1.65 Xba I fragment gave three bands corresponding to the products of other nick , the one in the 2.1 Eco R1 fragment.

The 0.5 Xba I fragment gave two bands of different molarity, although the orientation was opposite to that expected.

A summary of the molecular weights of the different fragments obtained after denaturation from the Eco R1 and Xba I fragments is shown in Fig. 34. The molecular weights are expressed as their equivalent in the double-stranded fragments to facilitate their interpretation.

Fig. 33. Electrophoretic analysis of denatured CaMV DNA fragments after digestion with Xba I. The Xba I fragments were isolated (Langridge), denatured by addition of alkali and electrophoresed in neutral agarose gels.

(a) 2.5 Xba I fragment

(1a) Denatured 2.5 Xba I fragment.

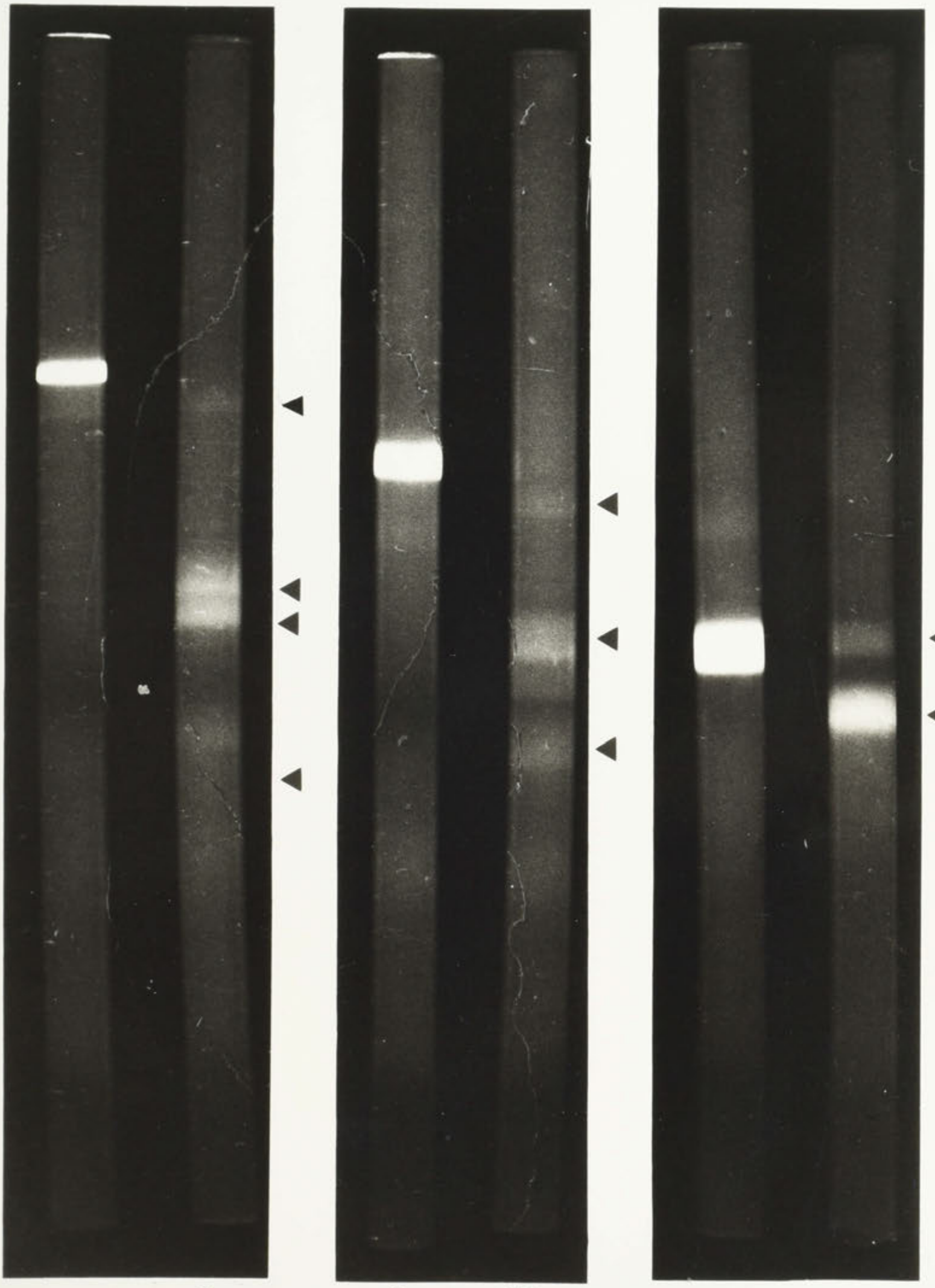
(b) 1.65 Xba I fragment.

(1b) Denatured 1.65 Xba I fragment.

(c) 0.5 Xba I fragment.

(1c) Denatured 0.5 Xba I fragment.

The black triangles indicate the bands obtained following denaturation.



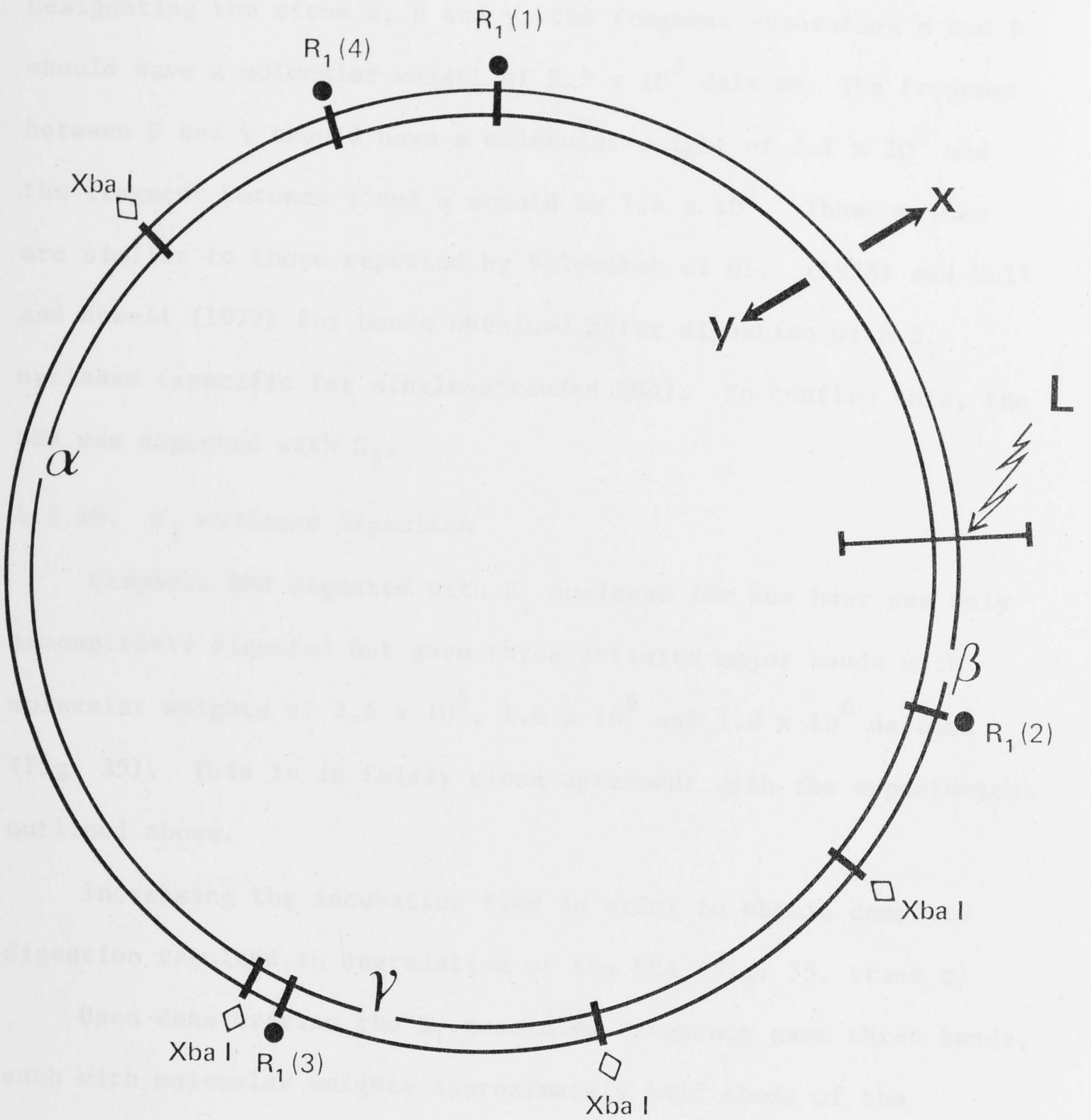
a 1a b 1b c 1c

Fig. 34. Map of the CaMV DNA showing single-stranded gaps with respect to the Eco R1 and Xba I sites. The single-stranded gaps are identified as α, β and γ. The DNA strands are identified as X (the outside circle) and Y (the inner circle). L refers to the most probable linearization point. The black dots mark the Eco R1 sites identified as R1 (1), R1 (2), R1 (3), R1 (4) and ($\diamondsuit$) the Xba I sites.

The table gives the molecular weights (x 10^{-6} daltons) of the double-stranded Eco R1 fragments (column 2) and the single-stranded fragments from strand X (col. 4) and strand Y (col. 5) after denaturating Eco R1 digested CaMV.

To facilitate interpretation the molecular weights of the denatured fragments (cols. 4 and 5) are given in double-stranded equivalents. Their actual expected molecular weight is half the value presented.

MAPPING OF SINGLE-STRANDED GAPS WITH RESPECT TO THE Eco R_1 AND Xba I SITES



Eco R_1 Sites	D-S Fragment		Strand X	Strand Y	Fragment missing
3–4	2.1	=	2.1	1.2+0.8	–
3–2	1.2	=	1.2	1.0	–0.2
2–1	1.5	=	1.4	1.5	–0.1
1–4	0.3	=	0.3	0.3	–

The distance between the nicks can be obtained from Fig. 32 based on the sizing done in alkaline gels with the Eco R1 fragments. Designating the sites α, β and γ, the fragment separating α and β should have a molecular weight of 2.5×10^6 daltons. The fragment between β and γ should have a molecular weight of 1.1×10^6 and the fragment between γ and α should be 1.4×10^6. These values are similar to those reported by Volovitch *et al.* (1978) and Hull and Howell (1978) for bands obtained after digestion with S_1 nuclease (specific for single-stranded DNA). To confirm this, the DNA was digested with S_1.

4.3.10. *S_1 nuclease digestion*

Campbell DNA digested with S_1 nuclease for one hour was only incompletely digested but gave three definite major bands with molecular weights of 2.4×10^6, 1.6×10^6 and 1.0×10^6 daltons (Fig. 35). This is in fairly close agreement with the expectations outlined above.

Increasing the incubation time in order to obtain complete digestion resulted in degradation of the DNA (Fig. 35, track c).

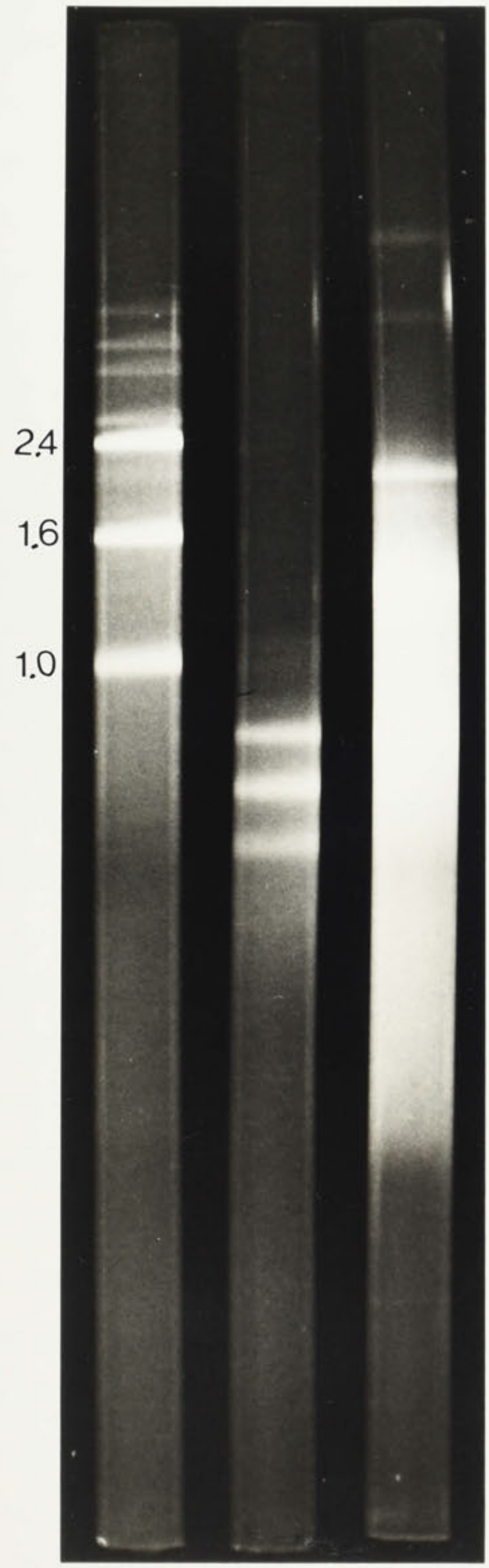
Upon denaturation the S_1 generated fragments gave three bands, each with molecular weights approximately half those of the respective major bands (Fig. 35, track b).

4.3.11. *Discussion*

The genomes of all three isolates used during this study each have three single-stranded nicks. These nicks, revealed by denaturation, are at fixed positions since in each strain they are at the same distance from one another. These gaps were mapped with respect to the Eco R1 and Xba I sites for the Campbell strain. In mapping the gaps with respect to Eco R1, there was no evidence of a

Fig. 35. Electrophoretic analysis of CaMV DNA digested with S_1 nuclease (a). The molecular weights corresponding to the major bands are in descending order 2.4, 1.6 and 1.0 x 10^6 daltons. The digestion was not complete and submolar bands are present. When incubation time was increased the DNA was degraded (c).

Track (b) shows the electrophoretic analysis of denatured CaMV DNA after digestion with S_1. The molecular weight of the bands is approximately half those of the double stranded bands (a).


2.4
1.6
1.0
a
b
c

fourth break present in some genome molecules and accounting for the fourth fragment (Fragment 3) found upon denaturation. This suggests that fragment 3 most probably comes from the linear molecules present in the native population of CaMV DNA. This hypothesis was also supported by comparisons between the strains. In newly isolated DNA of the strain New York, which normally had a low proportion of linear molecules, fragment 3 was hardly evident (Fig. 24). In contrast, in Campbell DNA with a high proportion of linear DNA, the band produced by fragment 3 was obvious. Hull and Howell (1978) proposed that the single-strand nick was located in position 0.24 in their map (corresponding to the site designated β in this work) and was the possible site of linearization of the molecules. The presence of submolar bands in their digestions with Eco R1 and Sal I supported this proposal since they pointed to a site very close to, or coincident with, this nick.

In my experiments with the Campbell isolate, the linearization point was tentatively mapped in the 1.5 Eco R1 band, separate from the Eco R1 site by a fragment of a molecular weight of 0.3×10^6 daltons (See discussion). The β single-stranded nick, was mapped at a distance equivalent to a fragment with a molecular weight of 0.1×10^6 from the same Eco R1 site. These facts locate the linearization point relatively close to, but not coincident with, the single-stranded nick, unless the latter is large enough to extend between the two points. There is no indication of the size of the nicks but it is unlikely that they are very extensive as judged by the molecular weight of the single-stranded fragments.

The hybridization patterns of the single-stranded fragments, (Fig. 30) make it obvious that band 1 is really a distinct double band formed by two fragments of very similar molecular weight with

a difference in molecular weight of less than 0.2×10^6 daltons (1a the heavier, 1b the lighter). This is also supported by the distribution of fragment sizes (Fig. 28) which shows 2 distinct peaks.

A model to account for this fact would locate the linearization point no more than 0.2×10^6 daltons from the single-stranded nick located in position β. This would also have to be the unique break in the strand X in Fig. 34. Locating the other two breaks in the complementary strand (Y) in their respective position the resulting products obtained when the circular and linear CaMV DNA molecules are denaturated can be deduced.

The circular molecules upon denaturation would produce three bands of lengths and molecular weights equivalent to those given by fragments 1a, 2 and 4.

The linear molecules would produce five bands, two from strand X and three from strand Y. The X strand would give a large fragment (1b) and a very small fragment (less than 0.2×10^6 daltons) beyond the resolution of the system. The Y strand would give a small fragment (Fragment 4) and Fragment 2 would be cut almost half-way giving two fragments of similar molecular weight (0.78 and 0.72×10^6) which would run as a slightly diffuse band in a gel and would have a broad normal distribution of sizes. These last two characteristic were those exhibited by Fragment 3.

A heterogenous population of DNA containing circular and linear molecules upon denaturation would give rise to a combination of the possibilities just mentioned; i.e., 6 bands: one too small to be detected; two heavy bands that would almost coelectrophorese (Fragment 1); one intermediate band (Fragment 2), one diffuse band directly related to the proportion of linear molecules in the

sample (Fragment 3) and one small band (Fragment 4).

This model would also account for the molar proportion in which the bands are found upon denaturation (Fig. 24) 1.27: 0.7: 0.25: 1.1 for fragments 1, 2, 3 and 4 respectively.

The linearization of the molecules might be related to the presence of ribonucleotides in the molecule, and this will be discussed in detail in Section 4.5.

It is not certain that the single-stranded breaks found in extracted DNA are present in the DNA in the CaMV virion; they may result from the extraction procedures as pointed out by Volovitch *et al.* (1978). They could be protected by a covalently bound protein lost during DNA extraction (Volovitch *et al.*, 1978). But whatever the reason for these gaps, they are highly conserved. They have been reported in every CaMV strain examined, located in exactly the same position, although some variability of restriction sites very close to the breaks has been encountered (Volovitch *et al.*, 1978). They might be of biological significance to CaMV, and this possibility is examined in the following section.

The complex hybridization pattern found using ^{32}P labelled cRNA copies for single and double-stranded fragments suggests that there are common sequences within the DNA genome. Homology between the 1.5 and 0.3 Eco R1 fragment was expected as pointed out in Section 4.2.2.1. This was found, but although the 0.3 Eco R1 fragment hybridized preferentially to the 1.5 Eco R1, it also hybridized with the 2.1 and 1.2 Eco R1 fragment. This could be due to amounts of contamination between the bands that could not be detected. Alternatively it could indicate common sequences within the genome. The presence of common sequences is also indicated by the amount of aggregation of DNA found every time that renaturation

was attempted. However, there was no evidence from analysis of restriction endonuclease sites of any repetition of DNA containing enzyme sites. This is not very surprising since in this study, as in the other published (Meagher *et al.*, 1977; Volovitch *et al.*, 1978; Hull and Howell, 1978) most of the enzymes used were those giving few cleavage sites. Also the genome of CaMV is fairly small and the regions of homology might also be very small, and would probably not contain recognition sites for the enzymes I used.

Regions of sequence homology are known in other viruses, such as Epstein-Barr virus and Herpes simplex, where two inverted repeats have been identified and characterized (Roizman, 1979). In Herpes simplex the sequence arrangement of the DNA consists of two covalently linked components of unique sequence, each one bracketed by a region and its inverted repeat, corresponding to 6% and 4.3% of the total DNA respectively (Roizman, 1979; Buchman *et al.*, 1978). The terminal 0.3% of the genome is repeated in every redundant sequence (Wilkie and Cortini, 1976). As a consequence of these inverted common sequences, viral DNA extracted from virions consist of four equimolar populations differing only in the orientation of the unique components relative to each other. When the DNA is cleaved by restriction endonucleases outside the common sequences, the fragments form three classes that differ in relative concentrations. Thus, the restriction fragments for some enzymes can vary from 0.25 M, 0.5 M to 1.0 M with respect to the intact DNA (Buchman *et al.*, 1978).

In Herpes simplex, where the terminal regions are inverted, internal recombination between the ends of the molecule and their inverted repeats might occur readily, leading to inversions of the unique components. The major difference observed between strains

was the presence or absence of particular restriction enzyme cleavage sites. In all likelihood a recombination event within a restriction site would generate a sequence which could no longer be recognised by the restriction enzyme. Likewise the occurrence of small deletions as found by Roizman (1979), which include the restriction site would abolish that site.

In Herpes simplex an understanding of the isomerization of the DNA has been facilitated by the fact that one virus particle is sufficient to initiate infection (Hoggan *et al.*, 1960). Moreover, the core of the capsid in the virus particle can only accommodate one molecule of viral DNA (Furlong *et al.*, 1972). These two facts led to the conclusion that the four isomers can arise from only one DNA molecule.

In CaMV there is no evidence that one particle can initiate infection. For most plant viruses many particles infections appear to be required before symptoms can develop. This, therefore, precludes the recognition of isomers within a viral population. The possibility of these being genome isomers in the CaMV genomes investigated might provide some explanations for the anomalies observed when mapping CaMV isolates with restriction endonucleases. For example, the fragments which result when CaMV is digested with Eco R1 do not occur in molar proportions. There are also indications of extra sites present in some part of the population, as occasionally faint bands of molecular weights of 0.9×10^6 daltons are seen, i.e., hybridization of Eco R1 fragments (Fig. 31). The variant of Cabb. B studied by Lebeurier *et al.* (1978) seems to have this site, which, in this study appears to be located in the 2.1 Eco R1 fragment. The fact that the CaMV DNA molecule is small does not leave room for dramatic changes that can be easily detected. But the fact that all the variability (new sites) as seen in the case

of Eco R1 are of the same magnitude (0.3×10^6 daltons) or a multiple of it might be of some significance. The fragments found in Eco R1 digests of different isolates are 2.1, 1.5, 1.2 and 0.3 $\times 10^6$ daltons, and occasionally a 0.9 and a 0.6 fragment were detected. Partial digests give fragments of 2.7, 3.0, 3.3×10^6 daltons. This might just be due to chance or might also suggest a mechanism of rearrangement of fragments within the molecule. This rearrangement or recombination would doubtless generate fragments having homologous sequences and could easily account for the cross-hybridization observed between fragments.

The existence of common sequences in CaMV especially, if they are small, could only be detected by direct nucleotide sequencing of the DNA genome, or at least of some specific fragments of it. If present, they would open new possibilities in understanding the origin of variation in CaMV, and even in its possible mechanism of multiplication which is still unknown.

The other striking characteristic of CaMV DNA, namely the presence of single-stranded breaks in the genome might also induce recombination within the viral population. Single-stranded gaps are known to exist in other viruses but CaMV seems to be unique in having three gaps distributed in specific sites within the genome.

Several viruses are known to contain single-stranded breaks within their genomes. For example Herpes simplex has alkali-susceptible regions, whose positions are still subject to controversy. Frenkel and Roizman (1972) concluded that the fragments found upon denaturation were not random in size, suggesting that the breaks have a specific location. On the other hand, Wilkie (1973) concluded that the DNA is randomly nicked and that the intact strands were not unique.

Single-stranded gaps have also been described in the phage T_5. This bacteriophage has a linear duplex DNA genome with one intact strand and with three to four uniquely positioned interruptions in the other strand (Bujard, 1969; Jaquemin-Sablon and Richardson, 1970; Hayward and Smith, 1972a, 1972b; Rhoades, 1977). The significance of the positions of these interruptions in T_5 is unclear. It has been postulated by various authors that these single-stranded nicks might:-

(a) Provide initiation points for DNA replication (Gilbert and Dressler, 1968).

(b) Serve as either initiation or termination points for transcription (Hayward and Smith, 1973).

(c) Act (at least for 1 of the nicks) as a signal to separate the two discrete steps occurring in the injection of T_5 DNA into a bacterial cell (Lanni, 1968).

(d) Provide foci for high levels of site specific recombination (Abelson and Thomas Jr., 1966).

There is no evidence to conclusively support one or other of these explanations to the exclusion of the others. Thus, the definite role, or roles, of single-stranded gaps in DNA genomes remains unknown.

In CaMV the three single-stranded nicks have been reported in all the strains examined so far (Volovitch *et al.*, 1978; Hull and Howell, 1978), with the exception of CM4-184 (Hull and Howell, 1978) where the γ site is missing; the absence of this site is probably due to a deletion in this region (Hull and Howell, 1978). This strain has also lost the ability to be transmitted by aphids (Lung and Pirone, 1973, 1974). A detailed comparison of this strain with strains containing the three single-stranded nicks is

an essential first step in attempting to understand the biological role of these single-stranded gaps. A full understanding of the seemingly essential nature of these gaps, be they concerned with replication, transcription, infection, recombination or some other function, will doubtless demand the isolation of additional strains missing these single-stranded nicks.

4.4. POSSIBLE BIOLOGICAL SIGNIFICANCE OF THE SINGLE-STRANDED GAPS IN THE GENOME OF CaMV

4.4.1. *Introduction*

As expressed before, the presence of fixed single-stranded gaps in all the strains of CaMV examined suggests that they might play an important role in the biology of CaMV.

The genomic DNA of the strain New York, has been cloned by Dr. J. Langridge in the *E. coli* plasmid pMB9, by linearizing the DNA with the enzyme Bam I and inserting this linear molecule into the Bam I site of pMB9. After excision from the plasmid and subsequent ligation, the now circular viral DNA had lost its infectivity, or at least the capacity to produce symptoms in turnip cv. Just Right, mustard cv. Tendergreen and rapeseed cv. Masowiecki.

Nicks that were present in the original CaMV genomic DNA will be repaired during amplification of the ligated pMB9/CaMV hybrid molecule within the *E. coli* host. This modification of the CaMV genome could be related to the loss of infectivity of the cloned DNA. This possibility was investigated by monitoring the infectivity and the presence or absence of gaps during different stages of the cloning process.

The infectivity was assessed by inoculating seedlings of both turnip and mustard under conditions specified in Materials and

Methods Chapter 3, Section 3.5.14.

The presence or absence of single-stranded gaps was determined by electrophoresing DNA denatured with alkali in neutral gels, as done during the mapping of these gaps. Any differences in the number or molecular weight of the fragments obtained upon denaturation would indicate changes in the positions of the nicks.

Also filling the gaps *in vitro* was attempted.

4.4.2. *Presence of gaps in the cloned DNA*

To verify the absence of gaps in the cloned DNA, newly cloned New York DNA was examined.

The cloned DNA, after being excised from the plasmid and ligated, consisted of a mixed population of monomeric and dimeric molecules of CaMV DNA (Fig. 36, track a, b). The monomers migrated in gels at the same rate as the native circular DNA. The dimers had a molecular weight twice that of native circular DNA. Of this DNA, 40 µg were used to inoculate 32 turnip seedlings and 2 µg to detect the presence or absence of single-stranded regions. None of the plants developed any symptoms within five weeks of inoculation, and the cloned DNA did not have any single-stranded regions. (Fig. 36, track c).

4.4.3. *Infectivity and presence of gaps in the DNA prior to multiplication in the bacteria*

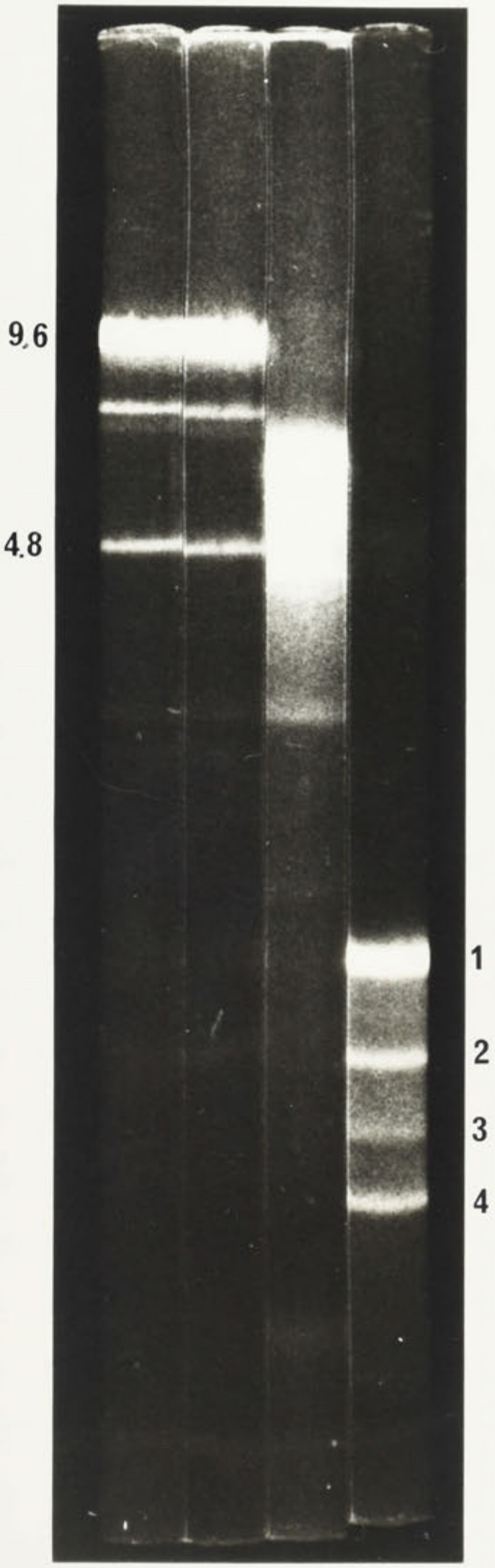
Having established that cloned CaMV had both lost infectivity and was devoid of single-stranded nicks, it was considered necessary to examine the CaMV DNA during the construction of the recombinant molecule. Samples were taken after each step in the cutting and ligation process (see Materials and Methods) and were checked for infectivity and presence of single-stranded gaps.

Fig. 36. Electrophoretic analysis of cloned CaMV DNA. CaMV DNA was cloned by Dr. J. Langridge in the *E. coli* plasmid pBM 9, by linearizing the DNA with enzyme Bam I and inserting this linear molecule into the Bam I site of pBM 9.

(a and b) show CaMV DNA after excision from the plasmid and ligation. The heavy top band corresponds to dimer molecules of CaMV DNA (molecular weight 9.6×10^6 daltons). The second band corresponds to supercoiled dimers and the third band correspond to CaMV DNA molecules (4.8×10^6 daltons).

(c) Cloned CaMV DNA cut from plasmid, ligated and denatured by addition of NaOH. No single-stranded gaps are present in the cloned DNA.

(d) Native CaMV DNA denatured by addition of alkali.


9.6
4.8
1
2
3
4
a b c d

Each sample contained 12 µg of freshly isolated New York DNA of which 10 µg were used to inoculate turnip and mustard seedlings and 2 µg were used for electrophoresis before and after denaturation.

The results of the infectivity tests and gel electrophoresis are presented in Table 4.4 and Fig. 37 respectively.

It is clear from Table 4.4 that ligation did not alter infectivity relative to that of untreated DNA. Track f in Fig. 37 shows that ligation was effective, since there was a band migrating faster. This band probably corresponds to coiled molecules which migrate faster than relaxed circles or linear molecules (Le Pecq, 1971).

Cut DNA was not infective and a low level of infectivity (±10%) was recovered following ligation of the Bam I cut molecule. This could be due to the fact that ligation might produce a high proportion of dimeric molecules, which would not be infective. If this was the case, infectivity should increase with greater concentration of inoculum, which would have greater absolute quantities of monomers. This hypothesis was tested using the same DNA preparation as before. It should be noted that the DNA was now 6 weeks old and because the DNA loses infectivity on storage, higher concentrations of native DNA were used as control.

From the results in Table 4.5, it can be deduced that the reduced level of infectivity following cutting and ligation of the DNA cannot be mitigated by increasing the DNA concentration. The process of cutting and ligation appears to cause a 90% loss in infectivity, whereas incubating DNA under ligation conditions alone had no influence.

The cut and ligated DNA has a lower proportion of the population with the original single-stranded gaps as shown in Fig. 37, track 1c.

Table 4.1. Infectivity of New York DNA at stages during the cloning process.

Treatment	Infected plants/ Inoculated plants		Electrophoresis results presented in Fig. 37	
	Turnip	Mustard[b]	untreated DNA	denatured DNA
1. Untreated DNA*	14/16	8/16	a	1a
2. DNA cut with Bam I	0/16	0/16	b	1b
3. DNA cut with BAM I and ligated	2/16	1/16	c	1c
4. Ligated DNA (no previous cutting)	13/16	7/12	f	1f
5. DNA + ligase buffer[c]	14/16	6/16	d	1d
6. DNA + ligase buffer[d] + heating	15/16	2/12	e	1e
7. DNA + ligase buffer[e] + heating + ligase	13/16	2/12	g	1g
8. Untreated DNA 30 µg/0.5 ml[a]	15/15	10/16		
9. Cloned DNA 40 µg/0.5 ml	0/32	-		Fig. 36

* DNA concentration 10 µg/0.5 ml unless specified.

(*a*) DNA at this concentration produced a few local lesions in turnip seedlings.

(*b*) In no experiments were all mustard seedlings infected. Turnip was more sensitive indicator and following this experiment all the infectivity tests were done using turnip.

(*c*), (*d*), (*e*). Indicates the different steps involved in the ligation of DNA, namely addition of ligase buffer (*c*), heating of the sample to 65°C (*c*), and addition of ligase (*e*) after it had been slowly cooled down to 4°C.

Fig. 37. Electrophoretic analysis of New York CaMV DNA. The presence or absence of single stranded gaps were monitored in the DNA during the cutting and ligation procedures prior to cloning. The top gels show DNA before denaturation and the bottom gels after denaturation, after each step of the process.

(a, 1a) Control. Native DNA.

(b, 1b) CaMV DNA after cleavage by Bam I.

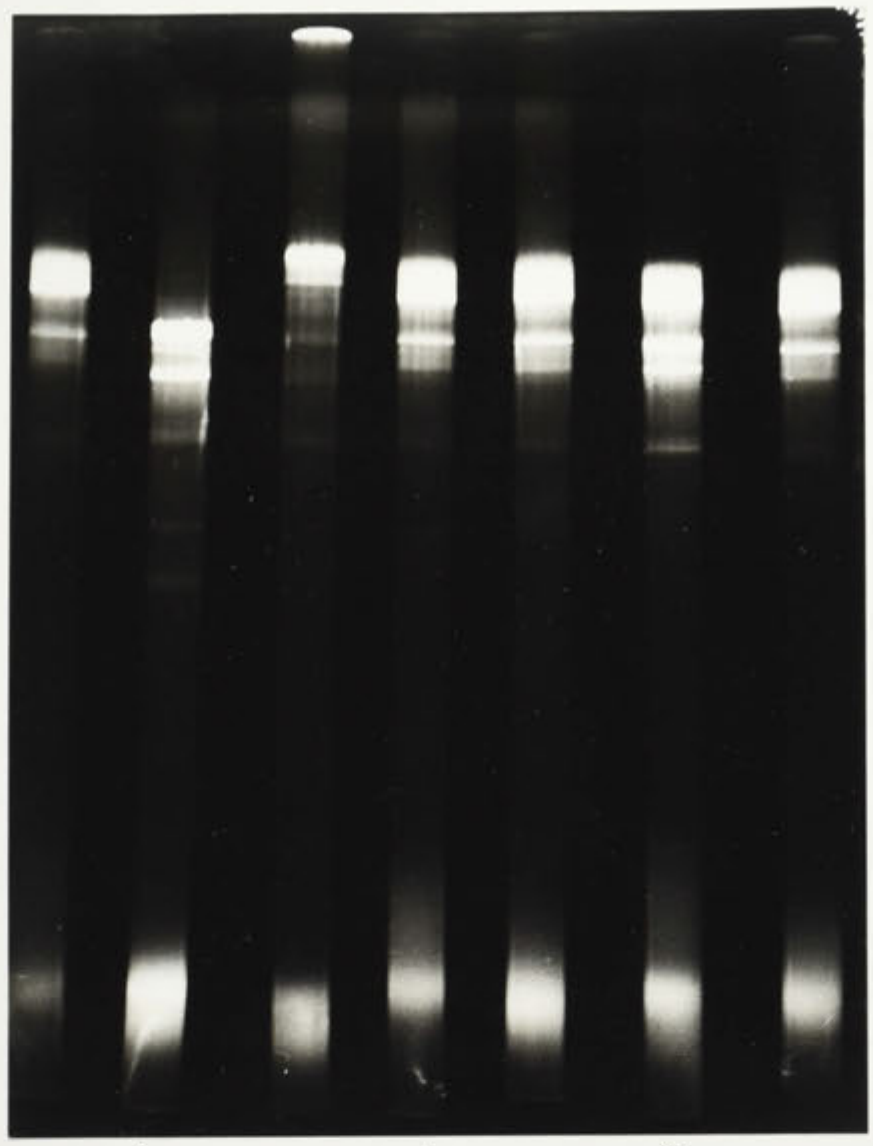
(c, 1c) CaMV DNA cut by Bam I and ligated. Note again that the top band corresponds to dimer molecules.

(d, 1d) DNA in ligase buffer.

(e, 1e) DNA in ligase buffer after heating at 65°C for 5 min.

(f, 1f) DNA ligated without previous cutting.

(g, 1g) DNA in ligase buffer, after heating, plus ligase (no ligation has taken place yet).


a b c d e f g

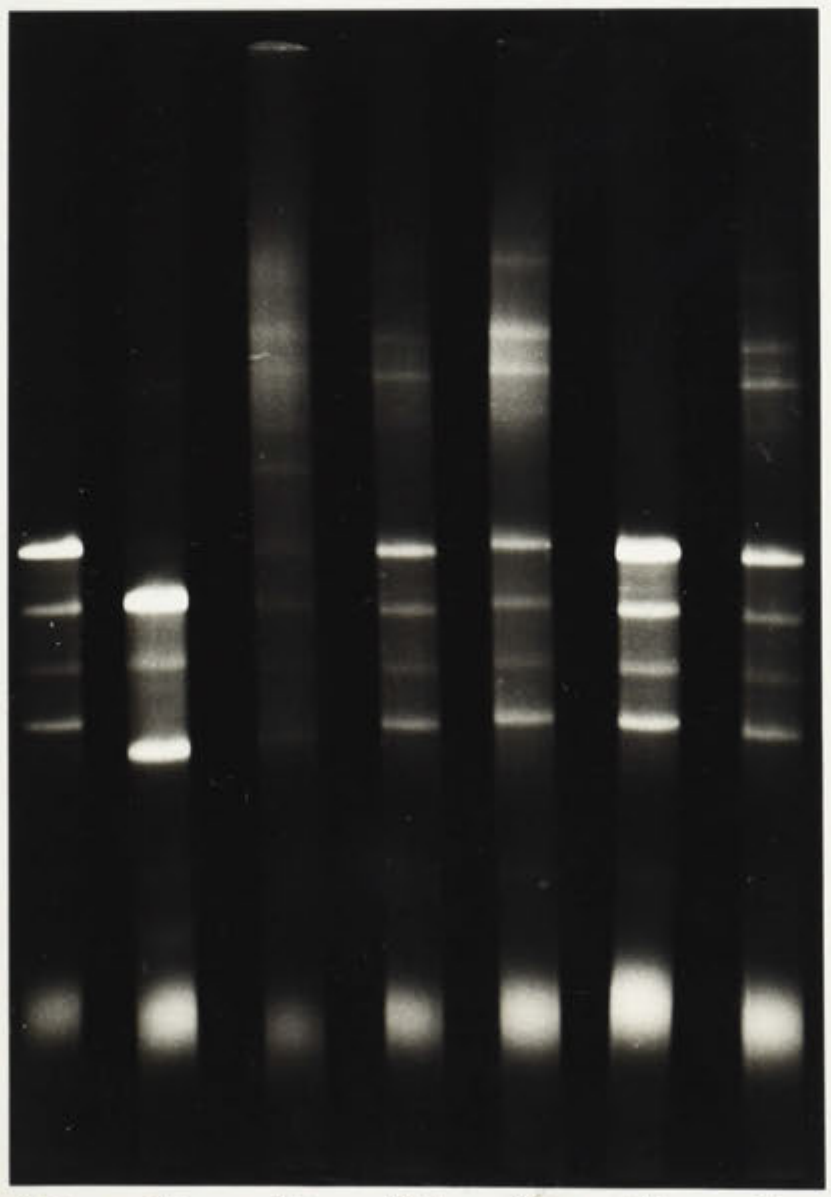

1a 1b 1c 1d 1e 1f 1g

Table 4.5. Effect of concentration on infectivity of cut and ligated New York DNA.

Treatment	Infected plants/Inoculated plants
Cut & ligated DNA 20 µg	1/16
Cut & ligated DNA 40 µg	2/16
Cut & ligated DNA 100 µg	1/16
Untreated DNA 40 µg	7/16

This could account for the low level of infectivity if the presence of single-stranded gaps is essential for infection. One way of testing this would be the filling of gaps *in vitro* and analysing the subsequent infectivity of the DNA, or obtaining cloned DNA in which the gaps have been preserved.

The first approach was attempted by treating the DNA with *E. coli* DNA polymerase in the presence of deoxyribonucleotides. Since no control or precedent for this procedure was known, the process was monitored by comparing native and denatured DNA by electrophoresis during the *in vitro* gap filling process. If any or all the gaps were filled, this could be detected by differences in the number and/or molecular weight of the fragments from the "filled" DNA relative to untreated DNA following denaturation. For example, if one of the nicks in the strand which originally had two was filled, after electrophoresis of the denatured DNA only 1 band should be visible, or 2 bands of similar molecular weight if there was different migration of the strands (Hayward, 1972).

The gels presented in Fig. 38, track b show that the DNA polymerase changed the pattern of the denatured DNA. The presence of new bands of intermediate molecular weight indicated that the DNA polymerase had exonuclease activity, thus precluding a meaningful comparison between native DNA and DNA without single stranded gaps produced by *in vitro* procedures.

4.4.4. *Discussion*

Cloning CaMV genomic DNA in a bacterial plasmid resulted in modifications in the structure of the DNA. The single-stranded nicks present in the native DNA were "repaired" during amplification. Since this resulted in loss of infectivity it also suggests a relation between the presence of gap and infectivity. Szeto *et al.* (1977) have reported loss of infectivity in CaMV DNA after cutting with the enzyme Sal I and subsequent ligation and of cloned CaMV DNA.

If CaMV is to be used as a vector for plant transformation, it is essential to establish the reason for the loss of infectivity. In a system for plant transformation the vector would need to be cleaved and ligated to insert foreign DNA into it. This vector would also need to be amplified in a convenient host, such as *E. coli* to provide adequate amounts of the vector. However, if the modifications produced in this process destroy the function of the vector as an "infectious" agent, different ways, or systems of amplification, must be sought.

There is also the possibility that the location of the site for insertion could be responsible for the subsequent lack of infectivity, and this can be altered by selecting different sites of insertion. For example, cutting the molecule at the Bam I site might damage the origin of replication. Alternative restriction sites need to be

Fig. 38. Electrophoretic analysis of CaMV DNA after treatment with DNA polymerase as specified in Materials and Methods. The effect of DNA polymerase was monitored by examining the DNA before and after denaturation.

(a) CaMV DNA after treatment with DNA polymerase.

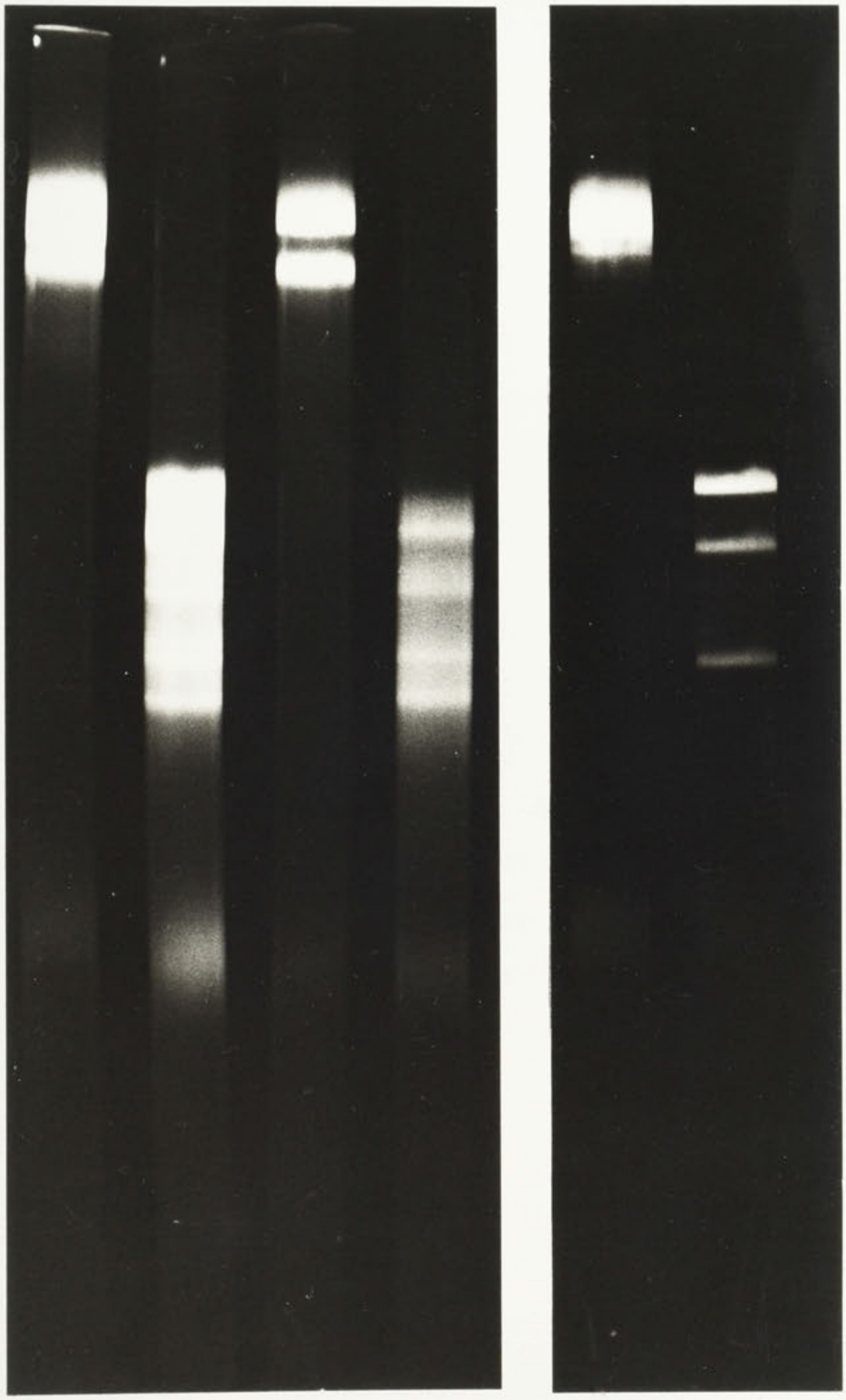
(b) Denatured CaMV DNA after treatment with DNA polymerase.

(c) CaMV DNA incubated with ribonuclease H before treatment with DNA polymerase.

(d) The same treatments as (c) plus denaturation.

(e) Native CaMV DNA.

(f) Denatured CaMV DNA.



a b c d e f

examined to determine whether the abolition of infectivity is a general or a specific problem.

4.5. PRESENCE OF RNA IN THE GENOME OF CaMV

4.5.1. *Introduction*

Hull and Shepherd (1977) reported the presence of a small amount of RNA in the CaMV genome. This RNA amounted to 0.6 nucleotides/100 nucleotides and is covalently linked to the genome (Hull and Shepherd, 1977; Shepherd, 1979). Thus there are approximately 180 ribonucleotides or 90 base pairs in the CaMV genome.

DNA polymerases are seemingly unable to attach directly to DNA and need an RNA primer sequence *in situ* on the DNA for initiation of transcription to occur (Scheckman *et al.*, 1974; Gefter, 1975; Brutlag *et al.*, 1971).

In some viruses with circular DNA genomes e.g., ϕx174 (Keller, 1972) the RNA primer is removed enzymatically when the DNA becomes circular, but in other virus and plasmid DNAs, such as those of T_4 (Speyer *et al.*, 1972) and Col E_1 (Williams *et al.*, 1973), the RNA that acts as primer remains in place. Hull and Shepherd (1977) argue that the RNA present in CaMV is probably an RNA primer remaining *in situ* on the DNA molecules.

The pattern of denaturation of the CaMV DNA is apparently the same, (Fig. 29, Chapter 4, Section 4.3.5.) whether the DNA is denatured by heating or by alkali. Therefore as alkali, but not heat, selectively hydrolyzes RNA (Yudkin and Offord, 1973) it implies either that ribonucleotides are not present in the isolate I examined or they are located at or near one or more of the single-stranded regions.

There is no direct indication of the possible location of the RNA in any isolate of CaMV, and assuming that RNA is present, several possibilities can be considered, taking into account all the data collected for CaMV. These possibilities are that:-

(a) RNA is located in one or all of the gaps. Thus the DNA would be resistant to S_1 nuclease digestion since this enzyme does not hydrolyze DNA-RNA hybrid regions. Also the denaturation products obtained by heating and alkaline treatment would be different.

(b) There is one double-stranded RNA region within the genome. This region would be susceptible to hydrolysis by pancreatic ribonucleases. CaMV DNA treated with pancreatic ribonuclease (ref. Section 4.11, Fig. 5) was not affected when examined by agarose gel electrophoresis (Fig. 5) in the proportion of circular and linear molecules recovered. If the RNA was all in one double-stranded RNA region, more linear molecules would probably have been found.

(c) RNA is located in the single-stranded regions. This would also give differences between denaturation accomplished by heating or by alkali. The alkali treatment should give more fragments (6).

(d) The RNA is located adjacent to the gaps in the same strands as the gaps, forming DNA-RNA hybrid regions.

Considering the amount of RNA in the molecule and the possibility of its distribution in 3 places, the difference in denaturation pattern obtained by heating or by alkali would be practically indistinguishable in an agarose gel system.

The enzyme RNase H purified from avian myeloblastosis virus specifically hydrolyses polyribonucleotides in DNA-RNA hybrids (Leis *et al.*, 1973, Stavrianopoulos and Chargaff, 1978). This property was utilized to search for the existence of DNA-RNA hybrid regions within the CaMV genome.

4.5.2. *Effect of RNase H on CaMV genome*

Since no DNA known to contain DNA-RNA hybrid regions was available to check the activity of RNase H, internal controls were done by comparing the DNA after incubation with RNase H (for 1 hour at 37°C), by electrophoresis, before and after denaturation (Fig 39). If the RNA in the molecule is the primer for replication its removal would affect replication and therefore infectivity. The infectivity of 35 µg of DNA, treated with RNase H was assessed in turnip seedlings. A treatment in which RNase A was used in place of RNase H was also included.

No changes in the denaturation pattern or in the morphology (linearization) of the native DNA were detected by gel electrophoresis following treatment with either RNase (Fig. 39). The infectivity, however, was greatly reduced in the presence of RNase A, compared with the other two treatments (Table 4.6).

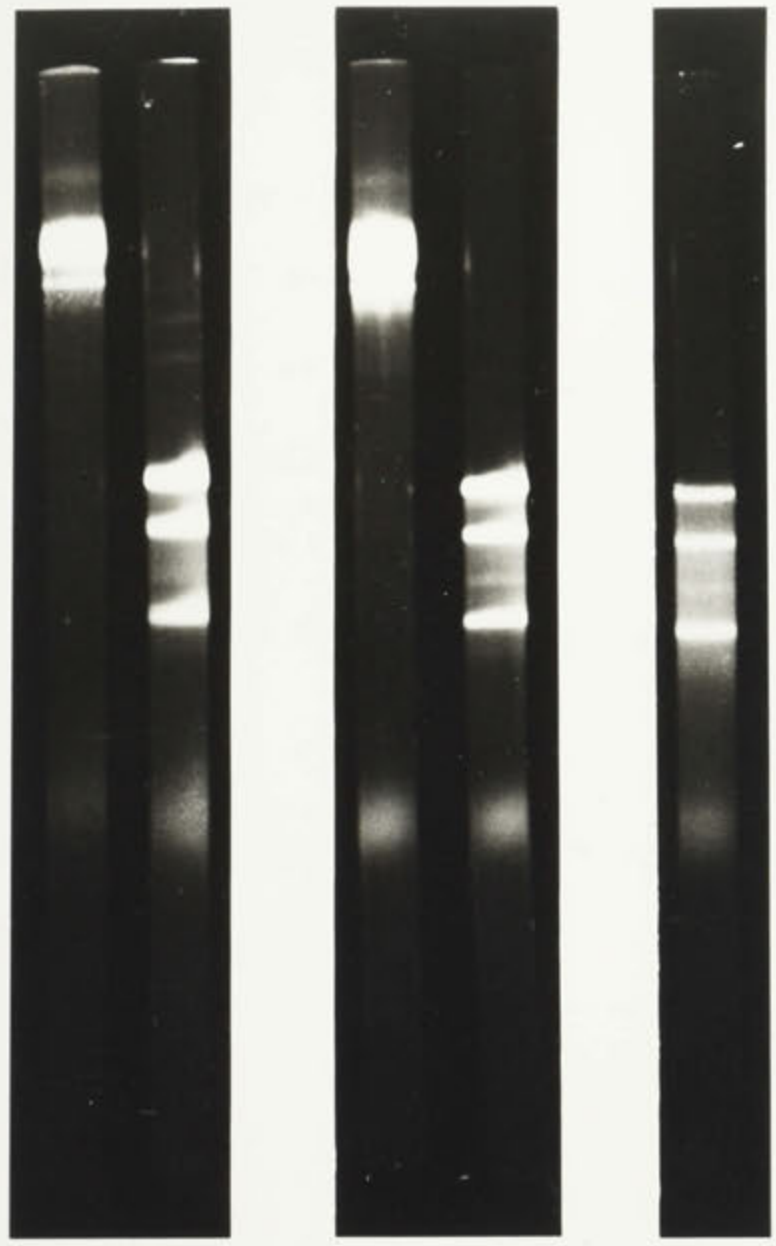
Table 4.6. Effect of ribonuclease H or pancreatic ribonuclease A on the infectivity of CaMV DNA.

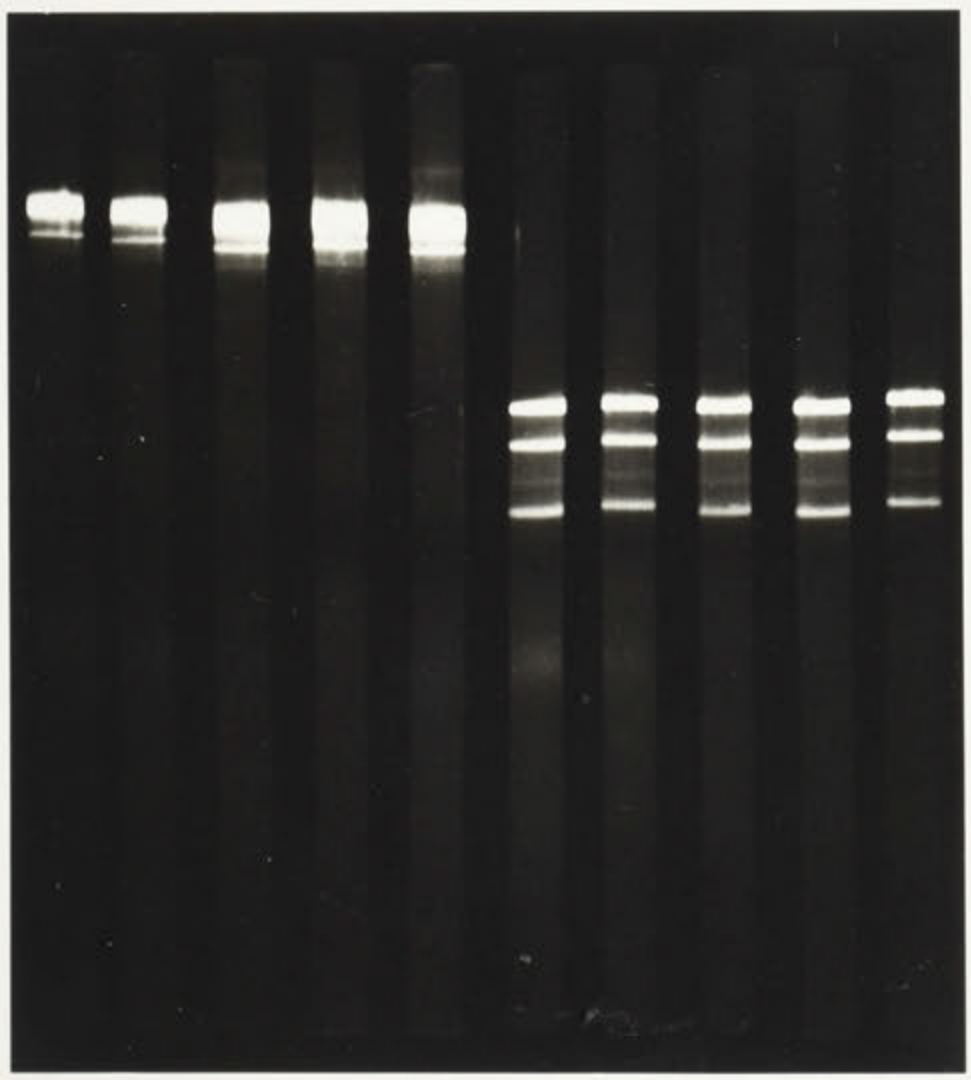
Treatment DNA conc. = 35 µg	Infected Plants/Inoculated Plants
DNA	30/32
DNA + RNase H	29/32
DNA + RNase A	9/32

Since this drastic effect of RNase A on infectivity (75% decrease) was unexpected, the procedure was repeated. To check the possibility that the RNase A solution was contaminated, a second source of RNase A, which is routinely used in preparation of RNA — DNA

Fig. 39. The top set of pictures show the electrophoretic analysis of CaMV DNA incubated with RNase H (a, 1a) and pancreatic RNase A (b, 1b) before and after denaturation, respectively. (c) Denatured CaMV DNA.

The bottom picture shows the electrophoretic analysis of CaMV DNA (a and 1a) incubated with RNase H, (b, 2b), pancreatic RNase A (c, 1c) (d, 1d) and pancreatic RNase T_1 (e, 1e) before and after denaturation, respectively. In all cases the DNA was incubated for 1 hr at 37° with the respective RNase, and it was denatured by the appropriate treatment just prior to electrophoresis.


a
1a
b
1b
c


a
b
c
d
e
1a
1b
1c
1d
1e

hybrids (gift of Dr. E.S. Dennis) was used. Also another sample was treated with pancreatic RNase T_1. The concentration of RNase A was increased to 4 µg/40 µg of DNA.

Again, there was no visible change in the DNA as judged by gel electrophoresis but the infectivity was reduced by RNase T_1, and completely abolished by RNase A (Table 4.7). Both sources of RNase A were effective.

Table 4.7. Effect of pancreatic RNase A, T_1 and RNase H in the infectivity of CaMV DNA.

Treatment	Plants infected/Plants inoculated
DNA 40 µg	24/24
RNase H	24/24
RNase T_1	17/24
RNase A	0/24
RNase (Dr. E.S. Dennis)	0/24

To determine if the reduction of infectivity in the DNA by RNase A was dependent on time of incubation, the DNA was treated with RNase A at 37°C for 1 min, 30 min, 1 hr, 3 hrs, then placed in ice and the turnip seedlings inoculated immediately. The results are presented in Table 4.8. Incubation beyond 30 min did not appear to further reduce infectivity.

Table 4.8. Effect of incubation time of RNase A treatments in the infectivity of CaMV DNA

Treatment	Incubation time	Infected Plants/Inoculated Plants
DNA 35 µg	-	16/16
DNA + RNase A	1 min	13/20
DNA + RNase A	30 min	2/16
DNA + RNase A	1 hr	3/20
DNA + RNase A	3 hrs	2/16

Also to verify that the decrease of infectivity was not due to an indirect effect of the RNase A on the host plant, a second set of samples were incubated under the same conditions, and then the samples were deproteinized by phenol-chloroform extraction, extracted with ether and passed through a molecular sieve. Unfortunately the DNA was lost during this procedure, so the effect of RNase A in the plants could not be assessed.

4.5.3. *Discussion*

The data presented in this section, supports earlier reports that RNA is present in CaMV genome where it might act as a primer for replication. The fact that infectivity is greatly reduced when the DNA is treated with RNase A suggests that the RNA is double-stranded, and pyrimidine rich, which RNase A specifically hydrolyses. The possibility that infectivity is reduced by a direct effect of RNase A cannot be ruled out since the appropriate controls were lost.

The fact that RNase T_1 also reduced infectivity, but only half as much as the RNase A, also suggests some effect of the nuclease on the host. RNase T_1 cleaves RNA to the 3' side of G residues and in plants the effect of RNase A and RNase T_1 might be expected to be similar, if the content of AU and CG bases in plant RNAs are not very different. The greater inhibition caused by RNase A is more probably due to its effects on the virus, which has only 90 ribonucleotide pairs, than the host.

Also the results given with different times of incubation with RNase A seem to support this hypothesis. Incubating the DNA for 1 min only halves the infectivity, in contrast with the 90% decrease after 30 min.

The absence of changes in the electrophoretic patterns of treated DNA does not support the idea that RNase A has a direct effect on the viral genome. If a double-stranded RNA fragment is present within the genomic DNA all the circular molecules would have linearized. This assumes, of course, that this region is not protected in some way from the action of nucleases, and that all virions contain double-stranded DNA.

The most probable linearization point was located in the 1.5 Eco R1 fragment, which is not cleaved by any of the restriction endonucleases used in this study (Fig. 17). As discussed before this might imply a protection mechanism against restriction endonuclease cleavage that might also include protection against RNases. Another way of protection might be related to the geometrical configuration of the molecules. A question still not resolved in CaMV is the differences in electrophoretic migration of the molecules. These are not caused by differences in molecular weight but by

different degrees of "twistedness" of the molecules (Hull and Shepherd, 1977; Civerolo and Lawson, 1978; Meagher *et al.*, 1977; Volovitch *et al.*, 1978). Now that the presence of single-stranded gaps has been established, it is more difficult to understand this behaviour. Normally the presence of just one nick in a strand is sufficient to relax supercoiled molecules by generating a swivel in the complementary strand at a site in the helix opposite to the scission (Vinograd and Lebowitz, 1966). The presence of three gaps involving both strands would presumably result in relaxed circles, but in CaMV several degrees of coiling are present. This twistedness of the molecule is common enough to form definite classes that form discrete bands when electrophoresed in gels.

The presence of double-stranded RNA might also offer an explanation for the lack of infectivity of the linear molecules in native populations found by Hull and Shepherd (1977). If the molecules linearize due to hydrolysis of the RNA, which is required to initiate replication, they would be unable to multiply.

The RNA might be hydrolised by contaminant ribonucleases and this would cause the gradual loss of infectivity in storage. Ribonucleases are commonly present in glassware, hands of operators, solutions etc. Unless special precautions are taken, such as sterilization of glassware and/or buffer, acid or alkali washing of glass and instruments, the ribonucleases can affect the preparations.

The linearization point was mapped for the strain Campbell, and as discussed before, might explain the hybridization pattern obtained for fragments derived from denaturation by alkali. The same arguments are valid if RNA is located at this point. Also this would explain the apparent lack of difference between denaturation

by heating and by alkali. The difference in molecular weight of fragment 1a x 1b resolved in fragment 1 in Fig.30 is too small to be visible in agarose gels, but the difference might be detected by comparing the hybridization patterns of fragments obtained by the two methods. Hybridization techniques are more sensitive than gel electrophoresis and could resolve the double band of fragment 1. These should be present only in DNA denatured by alkali but not in those denatured by heating in a homogeneous circular population. But, once again, the presence of native linear DNA would interfere with the results.

The loss of infectivity of the DNA when cut with Bam I might also be related to the location of the RNA. The cleavage site for Bam I is relatively close to the linearization point (0.5×10^6 daltons). Opening the molecule at this point might expose the RNA to hydrolysis by nucleases.

In a recent meeting on "Genome Organization and Expression in Plants" at Edinburgh, Scotland (1979), it was reported (Rudi Appels, personal communication) that CaMV DNA cloned using the Sal I endonuclease site was infective after excision from the plasmid into which it was cloned and then subsequently ligated. Presumably, this DNA would not have single-stranded gaps, because they would have been "filled" during cloning. This result contrasts with that of Szeto *et al.* (1977) who reported loss of infectivity in CaMV which had been cloned using the Sal I site.

However, if infectivity is retained following cloning it would imply that there is no relationship between the presence of single-stranded nicks and infectivity. The role of the nicks is still unknown and open to speculation.

The infectivity of DNA of isolate New York, cut with Sal I and ligated, has been tested (in collaboration with Dr. R.D. Brock). In a preliminary test, the DNA was 100% infective as linear and ligated molecules in comparison with the control. No cloned DNA is available yet for comparative studies.

The Sal I site is well separated from the Bam I site (0.9 x 10^6) and from the linearization point, as established during the mapping of the isolates. Thus the cleavage of the DNA at this point perhaps does not expose the postulated RNA primer and thus replication of the molecule is not impaired.

CHAPTER 5.

CULTURE OF PLANT CELLS

5.1. INTRODUCTION

As I mentioned in Chapter 1, a system which is to be used for plant transformation, needs an appropriate recipient for the novel genetic material.

The ideal recipient is most likely to be cultured plant cells, which can be grown in large numbers under defined conditions. For a number of plant species, cultured cells seem to be totipotent, and thus genetically changed cells can be grown into mature plants and tested.

An ideal system would consist of:

(a) A procedure to isolate viable plant protoplasts to take up the novel DNA, or the vector containing the novel genes.

(b) Treated protoplasts which could readily regenerate their cell walls, divide, grow and establish cell cultures.

(c) Cell cultures which would grow in liquid suspension cultures and provide large populations of cells on which selective pressures can be applied. This selective pressure would allow recovery of transformants that subsequently could be increased by serial transfers. Liquid suspension cultures offer the advantage of homogeneity and repeatable measurement of growth rate.

(d) New plants which would be regenerated from the transformed cells and tested for the effects and expression of the new genes.

In this study, the possibilities of establishing a reliable cell tissue culture system to act as a recipient for a potential vector based on CaMV were explored.

As mentioned before CaMV has a restricted host range, infecting mainly members of the Crucifereae. The first species selected for establishment of tissue culture were those providing the best hosts for the virus.

5.1.1. *Tissue culture of different species of the genus* Brassica

The use of tissue culture has been limited to only a few species within the genus *Brassica*.

In vitro bud formation from excised stem segments has been observed in *Brassica oleracea* var. *botrytis* (Margara, 1969) and *B. oleracea* cv. Krasa (Horák *et al.*, 1975). Plants have been regenerated from stem explants of *B. oleracea* (Lustinec and Horák, 1970), rapeseed *B. napus* cv. Zephyr (Kartha *et al.*, 1974) and Chinese Kale, (*B. alboglabra* Bailey) (Zee and Lui, 1977).

Radwan (1976) reported successful establishment of callus and suspension cultures of rapeseed, but no details on growth rates or regeneration potential of the cultures was mentioned.

Haploid cultures have been established by anther culture, and haploid plants regenerated from such cultures in rapeseed (Thomas and Wenzel, 1975; Stringam, 1977), kale (Kameya and Hinata, 1970), turnip rape (Keller *et al.*, 1975).

5.1.2. *Isolation and culture of protoplasts from plants of the genus* Brassica

Kartha *et al.* (1974) succeeded in regenerating complete plants from protoplasts of *B. napus* cv. Zephyr. Quazi (1975) isolated protoplast from six different *Brassica* cultivars and observed that in culture the protoplasts underwent the first mitotic division but then ceased dividing. Thomas *et al.* (1976) reported the regeneration

of plantlets from protoplasts obtained from haploid plants of rape. The haploid plants had been obtained from embryoids induced in cultured anthers.

In my study, I tried to establish callus cultures and then suspension cultures of several species of *Brassica*.

The ability of plants to regenerate from callus varies from one species to another and from one cultivar to another within a single species (Scowcroft, 1977). Therefore, different species which had previously been successfully tested as hosts of CaMV were used as starting materials.

Protoplasts were also isolated and cultured from a diversity of *Brassica* sp., both virus-free and plants infected with CaMV, to determine whether protoplasts from such infected plants were different from those isolated from uninfected plants. Extracts of such protoplasts were also tested by inoculation to plants.

In epidermal cells of CaMV-infected plants inclusion bodies, that stain with 1% phloxine, can be seen (Shepherd, 1976). Therefore protoplasts isolated from *Brassica* sp. infected with CaMV were stained with phloxine and examined microscopically for inclusion bodies.

Since the culture of *Brassica* spp. proved difficult other alternative hosts were also established in tissue culture.

5.1.3. *Tissue culture of* Nicotiana clevelandii

Nicotiana spp., especially *N. tabaccum*, are probably the species that have been most fully studied for their ability to form plant tissue cultures (Carlson *et al.*, 1972; Melchers, 1977; Kao, 1977). *N. clevelandii* has been reported to be a host of CaMV and hence could perhaps be the ideal plant system for transformation. The feasibility of culturing tissues of this species was also explored.

5.1.4. *Culture of* Arabidopsis thaliana *tissues.*

Arabidopsis is one of the crucifers that has been used in tissue culture studies (Negrutiu, 1975, 1976). Protoplasts have been isolated, cultured and plantlets regenerated (Gresshoff, 1976) and furthermore there are also reports (Gleba and Hoffman, 1978) of hybrid cell lines produced by protoplast fusion of *A. thaliana* and *Brassica campestris*. This hybrid line regenerated callus and had been maintained in culture for at least seven months (Gleba and Hoffman, 1978) although no organogenesis was reported.

A. thaliana had been tested successfully as a host for CaMV (Chapter 3) thus, it was also considered as a potential recipient of genetic material.

5.2. MATERIALS AND METHODS

5.2.1. *Establishment of culture of cell tissues*

The plant species used were rapeseed, *Brassica napus* cv. Turret, Zephyr, Target and Masowiecki, and turnip *Brassica rapa* cv. Just Right.

Cultures were initiated from germinating seeds. The seeds were surface-sterilized by washing with 10% sodium hypochlorite solution in a gyratory shaker. After 30 min. the seeds were rinsed five times with sterile distilled water and germinated in Petri dishes on different agar culture media.

The media utilized were: B5 (Gamborg *et al.*, 1968), M&S (Murashige and Skoog, 1962) and CS 5 (Gibson *et al.*, 1976). Cultures were incubated at 28-30°C in the dark.

The effects of several components and factors on the quality and growth rate of the callus was studied by modifying the media composition, or by addition of new ingredients.

The factors studied were:

(a) Level and sources of nitrogen in the media, achieved by altering the proportions of nitrates (potassium nitrate) and ammonium salts (ammonium nitrate, ammonium phosphate and urea).

(b) The final pH of the media, which was altered by adding 1 M KOH or 1 M HCl.

(c) Different levels of vitamins. The vitamins in CS 5 were replaced by those of B5. The effect of vitamin E, calcium pantothenate and biotin were also tested.

(d) Auxins and auxin levels. Stock solutions of 4.5 mM 2,4-dichlorophenoxyacetic acid (2,4-D); 5.3 mM para-chlorophenoxyacetic acid (pCPA); 1.95 mM 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and 2.85 mM indole butyric acid (IBA) were prepared and the appropriate amounts added to the media to give the desired final concentration.

(e) Cytokinins. Stock solutions of 2.32 mM 6-furfurylaminopurine (Kinetin); 2.22 mM 6-benzylamino-purine (BAP); 2.46 mM N^6-isopentenyl-aminopurine (2iP), and 2.28 mM Zeatin were prepared and added to the media to give the appropriate concentration.

The effect of media was assessed by comparing the growth colour and friability of the callus.

5.2.2. *Cell tissue culture of* Nicotiana clevelandii

Stem and petiole explants of *N. clevelandii* were placed on solid CS 5 containing 9 µM 2,4-D and 0.2 mM kinetin. Suspension cultures were established and maintained in liquid CS 5 by placing 50 ml suspension in 250 ml flasks on a gyratory shaker (100 rpm) at 28-30°C in the dark. Once established the cultures were subcultured once a week.

5.2.3. *Cell tissue culture of* Arabidopsis thaliana

Callus and suspension cultures were obtained from seeds of *A. thaliana* race Columbia following the procedure reported by Negrutiu *et al.* (1975 and 1976). Once suspension cultures were established they were transferred to CS 5 media with the same additives as used for *N. clevelandii*.

5.2.4. *Isolation and culture of protoplasts*

Leaf material was obtained from rapeseed, *B. napus* L. cv. Zephyr, Target, Masowiecki, Kitela, Ramses and several other selections* grown in a 3:1 mix of vermiculite and Perlite under controlled conditions. The temperature during their growth was 13/18°C with only natural light. Leaves were collected at different times during the year from plants at different stages of development.

In addition the rapeseed cultivars Zephyr, Target and Masowiecki and the turnip cv. Just Right were also grown in vermiculite at a light intensity of 4500 lux (16/8 h photoperiod). The temperature and relative humidity averaged 19-21°C and 40-45% respectively (Kartha *et al.*, 1974).

These same cultivars were also grown in a glasshouse, in regular glasshouse soil mix with temperatures varying from 10-25°C under natural light only. Mustard, *Brassica perviridis* cv. Tendergreen, was also grown in these conditions. Leaves were harvested before they were fully expanded from 6 weeks old plants.

Protoplasts were also isolated from leaves of cauliflower, *B. oleraceae*, broccoli and parsnip grown in local gardens.

In all the cases the leaves were sterilized in 70% ethanol for 60 seconds followed by immersion in 10% sodium hypochlorite containing

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* The leaves originated from a collection of cultivars and lines of rapeseed maintained for screening against fungal diseases. Leaves were provided by Dr. K. Helms.

a drop of detergent (Tween 20) as wetting agent. After 10 min. the leaves were rinsed in 5 changes of sterile distilled water. All these and subsequent operations were done aseptically in a laminar flow cabinet.

The lower epidermis was removed and the leaves cut into small pieces prior to placing them in conical flasks.

The medium employed for the isolation is summarized in Table 5.1. The enzymes were desalted by dialysis against several volumes of distilled water. The medium was sterilized by filtering through a 0.45 µm Millipore filter.

25-35 mls of medium were added to 1 to 3 gms of stripped leaves which were infiltrated *in vacuo* for 2 min and then incubated in the dark in a gyratory shaker (50-70 rpm) for 3-4 hrs at room temperature.

Periodically samples were examined under a microscope to monitor the release of protoplasts. After most of the cells had become protoplasts the enzyme-protoplast mix was passed through a fine mesh sieve to remove undigested material. The protoplasts were then washed 4-5 times by gentle centrigugation (100 x g for 2 min.) and then resuspended in 5 ml of culture medium (Table 5.1) lacking the growth regulators (Kartha *et al.*, 1974).

The protoplasts were then resuspended in culture media containing growth regulators to give a final concentration of 2-5 x 10^5 protoplast/ml. Droplets (50 µl) of the suspension were dispensed in sterile 3.5 x 1 cm plastic Petri dishes. The dishes were sealed with adhesive tape and incubated in humidity chambers at room temperature (23°C).

The culturing media normally used was that of Kartha *et al.* (1974) but several modifications were introduced in an attempt to induce protoplasts to divide and multiply.

Table 5.1. Media used in the isolation and culture of *Brassica* sp. mesophyll protoplast.

Isolation Medium	g/100 ml
Driselase (Kyowa, Hakko Kogyo Co. Ltd.)	2.0
Cellulase "Onozuka" (All Japan Co. Ltd.)	2.0
sorbitol	4.55
mannitol	4.55
$CaCl_2.2H_2O$	0.0875
$CaH_4(PO_4)_2.H_2O$	0.0125
pH	6.2
Culture medium (Kartha *et al.*, 1974)	
B5* salts, trace elements and vitamins	-
sorbitol	4.55
mannitol	4.55
D-glucose	0.25
D-ribose	0.0125
N-Z-amine (Sheffield)	0.0150
$CaCl_2.2H_2O$	0.0875
2,4-D	2.3 µM
BAP	1 µM
NAA	1 µM
pH	5.8

* Gamborg *et al.* 1968.

The modification used at different times were:

(a) Changes of carbon sources: mannitol and sorbitol were replaced by glucose to give the same molarity (0.5 M). Small amounts of sucrose (25 mg/100 ml) were also added.

(b) Changes in type and concentration of growth regulator: different concentrations of 2,4-D, (4.5 mM, 2.2 mM, 1.1 mM) were used. BAP was replaced by zeatin at the same concentration.

(c) Addition of coconut milk in concentrations varying from 5 to 10%.

(d) Addition of 5 to 10% v/v turnip, broccoli, cauliflower extracts: These extracts were prepared by boiling 200 gms of turnip, broccoli or cauliflower in 1 litre of distilled water. After filtering the liquid it was sterilized by autoclaving and added to the media instead of, or in addition to, coconut milk.

5.2.5. *Isolation of protoplasts from CaMV infected plants*

The procedure for isolation was as previously outlined, but the epidermal strips removed from the leaves were also digested. After release the protoplasts were rinsed twice in the culture medium without hormones. Afterwards an aliquot of the preparation was resuspended for 1 min. in 5 ml of the same media to which 1% of phloxine dissolved in mannitol-sorbitol was added. Following five to six washes with the same media without phloxine. The protoplasts were observed under the microscope to check for the presence of inclusion bodies.

The rest of the sample was treated as usual, but after washing the protoplasts were resuspended in 0.1 M potassium phosphate buffer pH 7.2. This caused the protoplasts to burst and the solution was

used to inoculate turnip, mustard and rapeseed seedlings to check the infectivity of the virus.

5.3. RESULTS

5.3.1. *Culture of* Brassica *tissues; effect of composition of media*

The seeds of the varieties used germinated within 3-8 days and shortly afterwards callus formed on the cotyledons, radicles and hypocotyls. Pieces of this callus were transferred to new plates containing the same media.

After a few days it was apparent that the growth of the callus placed in M & S and CS 5 media was better than that of B5. This last media was not used for subsequent experiments. The rate of growth of the callus in all cases was very slow and the callus was hard and rubbery. To try to improve the callus viability the media were modified.

5.3.1.1. Amounts and sources of nitrogen

M & S and CS 5 were supplemented with 0.2 mM [N] in the form of ammonium phosphate, or 0.5 mM [N] in the form of ammonium nitrate or urea, or 1.25 mM [N] in the form of potassium nitrate. In all the treatments the addition of extra N was deleterious to the callus formation and growth. This was not unexpected because both M & S and CS 5 contain 60 mM N.

Growth was also poor when the proportion of major salts in CS 5 was reduced by half.

5.3.1.2. Effect of pH

The growth and quality of callus was best at pH 6. At pH 7.0 or pH 8.0 the callus was very hard and brown.

5.3.1.3. Amounts of vitamins

Several combinations of vitamins were tested. The best callus was obtained in CS 5 when the vitamins of this media were replaced by those of B5.

Addition of vitamin E (1 mg/l) was also beneficial. By contrast, calcium pantothenate and biotin either showed no effect or reduced growth rate.

5.3.1.4. Auxin and auxin concentrations

The effect of auxins was different for each species. In turnip pCPA at a concentration of 5.3 mM and 2.7 mM produced better callus than 2,4-D at concentration of 2.2 mM or 1.1 mM, but still the growth was very slow.

In rapeseed, the cultivar Turret produced callus which was more viable and grew at a faster rate than the other cultivar. Subsequently callus cultures of the other cultivars were abandoned.

In Turret the best callus was produced in the presence of 0.24 mM 2,4,5-T. The second best treatment was given by a low concentration of 2,4-D (0.55 mM). IBA was always deleterious, producing hard brown callus.

5.3.1.5. Cytokinin concentrations

In general larger amounts of cytokinins produced hard rubbery callus. Very small amounts of BAP (0.5 and 0.25 mM) increased the growth rate of the callus but did not improve its quality.

The best callus was obtained by using a combination of low levels of both kinetin (0.6 mM) and 2,4,5-T or 2,4-D

Fig. 40 presents a summary of the best treatments obtained in the different media used to culture rapeseed Turret.

Fig. 40. Comparative callus growth obtained with different levels and sources of auxins and cytokinins, and different vitamins with the rapeseed (*Brassica napus*) cv. Turret cultured in CS 5 medium (Gibson *et al.*, 1976). The CS 5 vitamins were replaced for those of B5 (Gamborg *et al.*, 1968). The treatments shown in the picture are:

A_1 = CS 5 + 0.55 mM 2,4-D

A_2 = CS 5 + 0.55 mM 2,4-D + 0.6 mM kinetin

A_3 = CS 5 + 0.55 mM 2,4-D + 1 mg/l Vitamin E

A_4 = CS 5 + 0.55 mM 2,4-D + 0.6 mM kinetin + 1 mg/l Vitamin E.

B_1 = CS 5 + 0.65 mM pCPA

B_2 = CS 5 + 0.65 mM pCPA + 0.6 mM kinetin

B_3 = CS 5 + 0.65 mM pCPA + 1 mg/l Vitamin E

B_4 = CS 5 + 0.65 mM pCPA + 0.6 mM kinetin + 1 mg/l Vitamin E.

C_1 = CS 5 + 0.24 mM 2,4,5-T

C_2 = CS 5 + 0.24 mM 2,4,5-T + 0.6 mM kinetin

C_3 = CS 5 + 0.24 mM 2,4,5-T + 1 mg/l Vitamin E

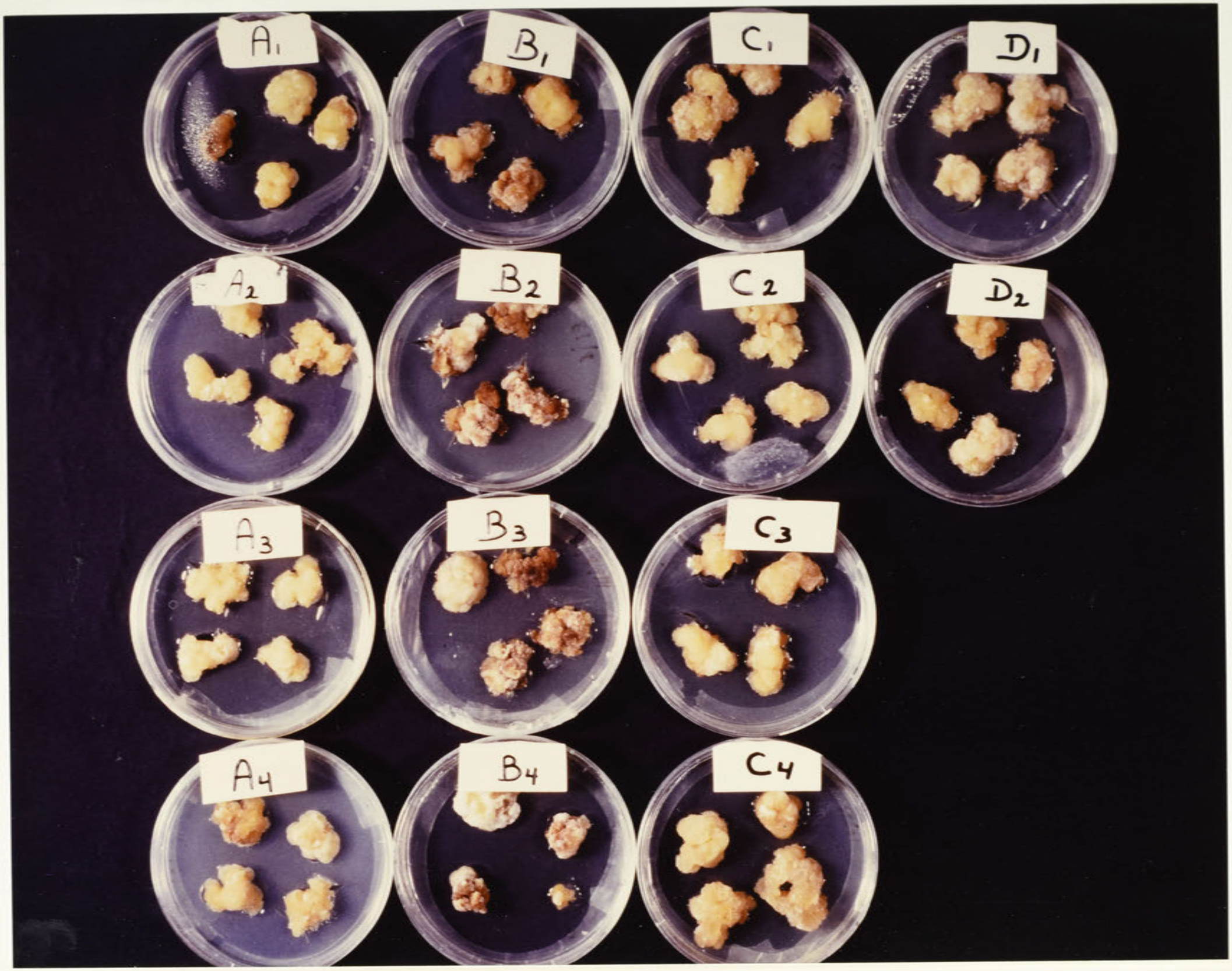
C_4 = CS 5 + 0.24 mM 2,4,5-T + 0.6 mM Kinetin + 1 mg/l Vitamin E.

D_1 = CS 5 + 1.95 mM 2,4,5-T

D_2 = CS 5 + 1.95 mM 2,4,5-T + 0.6 mM kinetin

D_3 = CS 5 + 1 mg/l Vitamin E.

The best treatments were C_4, C_2, A_2 and A_4.


A1
B1
C1
D1
A2
B2
C2
D2
A3
B3
C3
A4
B4
C4

5.3.1.6. Suspension culture of *Brassica*

The best callus was obtained from the rapeseed cv. Turret when grown in CS 5 at pH 6.0 in which the vitamins had been substituted by those of B5 and to which 0.24 mM 2,4,5-T or 0.55 mM 2,4-D, 0.6 mM kinetin and 1 mg/lt of vitamin E had been added. The callus was soft and relatively friable, white to light yellow in colour with relatively good growth (Fig. 40, C_4).

When this material was placed in the same media, but without agar, to establish suspension culture the growth was slow and the callus formed aggregates. When disaggregated by mechanical means growth ceased.

5.3.2. *Cultures of* Nicotiana clevelandii *cells*

Tissue cultures of *N. clevelandii* were easily established. Soft, light colour friable callus, grew at a greater rate on CS 5 in agar (Gibson *et al.*, 1976). After 6 transfers, pieces of callus were disaggregated and placed in CS 5 liquid media. The rate of growth was estimated using a Klett-Summerson photoelectric colorimeter which measures the effect of turbidity on optical density. The turbidity of the cultures doubled within 48 hrs which corresponds to a doubling of cell number (W.R. Scowcroft - personal communication).

5.3.3. *Cultures of* Arabidopsis thaliana *cells*

A. thaliana also callused easily and suspension cultures were readily established with cells forming minuscule aggregates. These cultures were later transferred to CS 5 and selected to eliminate the aggregates until true cell suspensions were obtained.

5.3.4. *Isolation and culture of* Brassica *protoplasts*

Protoplasts were readily isolated from all the species of *Brassica* used. These protoplasts were spherical with their chloroplasts evenly

distributed (Fig. 42) and showed active cytoplasmic streaming. None of the media, and modifications introduced, induced the protoplasts to divide and form colonies. Usually cell walls regenerated as indicated by changes in the shape of the protoplast. The cultures were maintained free of contaminants for up to six weeks, and occasionally one or two divisions were observed. In no instance did microcalli form.

5.3.5. *Isolation of protoplasts from* Brassica *sp. infected with CaMV*

Protoplasts were isolated and inclusion bodies were clearly visible in protoplasts stained with phloxine. Fig. 41 shows epidermal strips from healthy and CaMV infected plants and the protoplasts subsequently isolated from them.

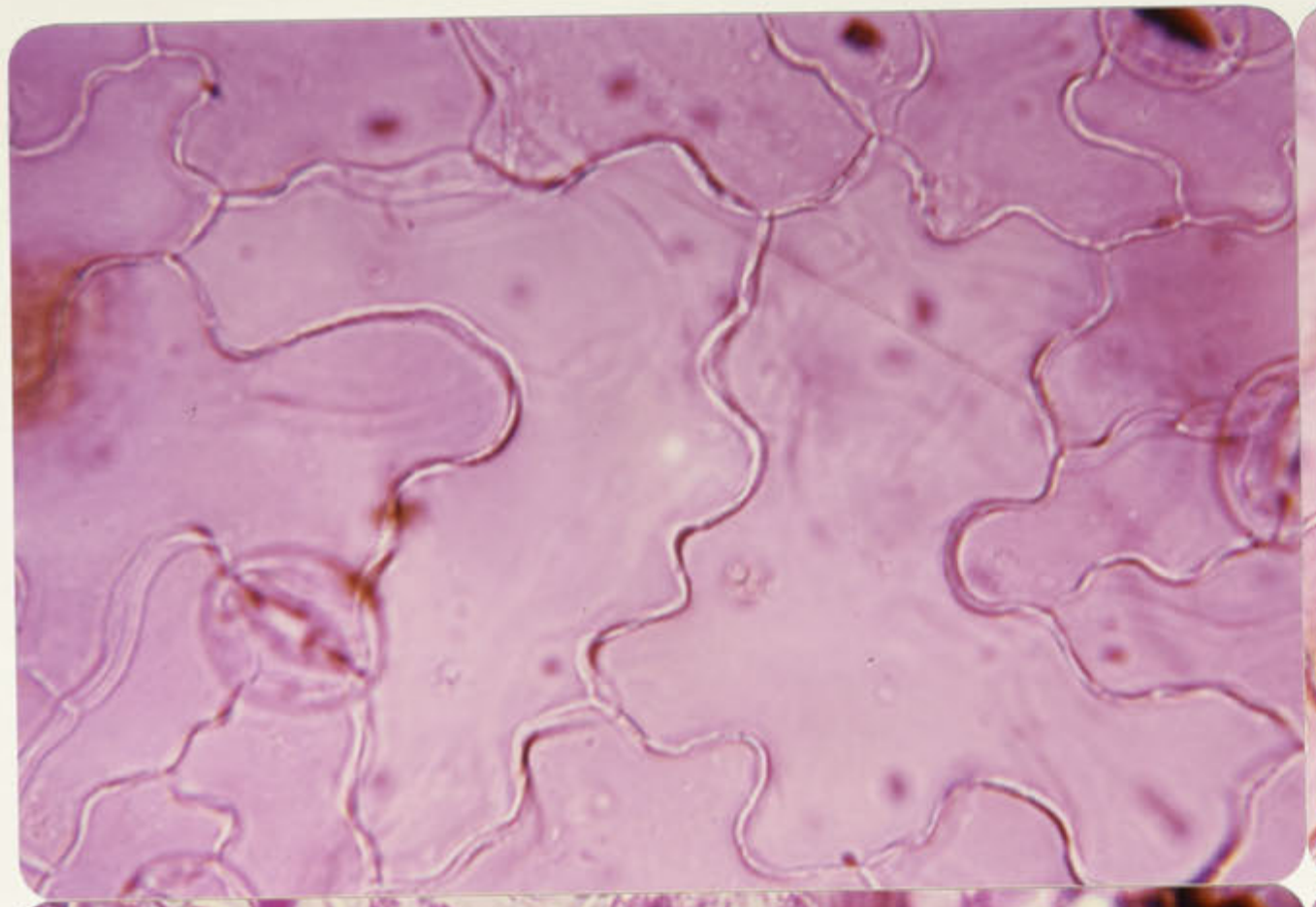
The protoplast isolation procedure did not alter the infectivity of the virus; infectivity in test plants reached 100%.

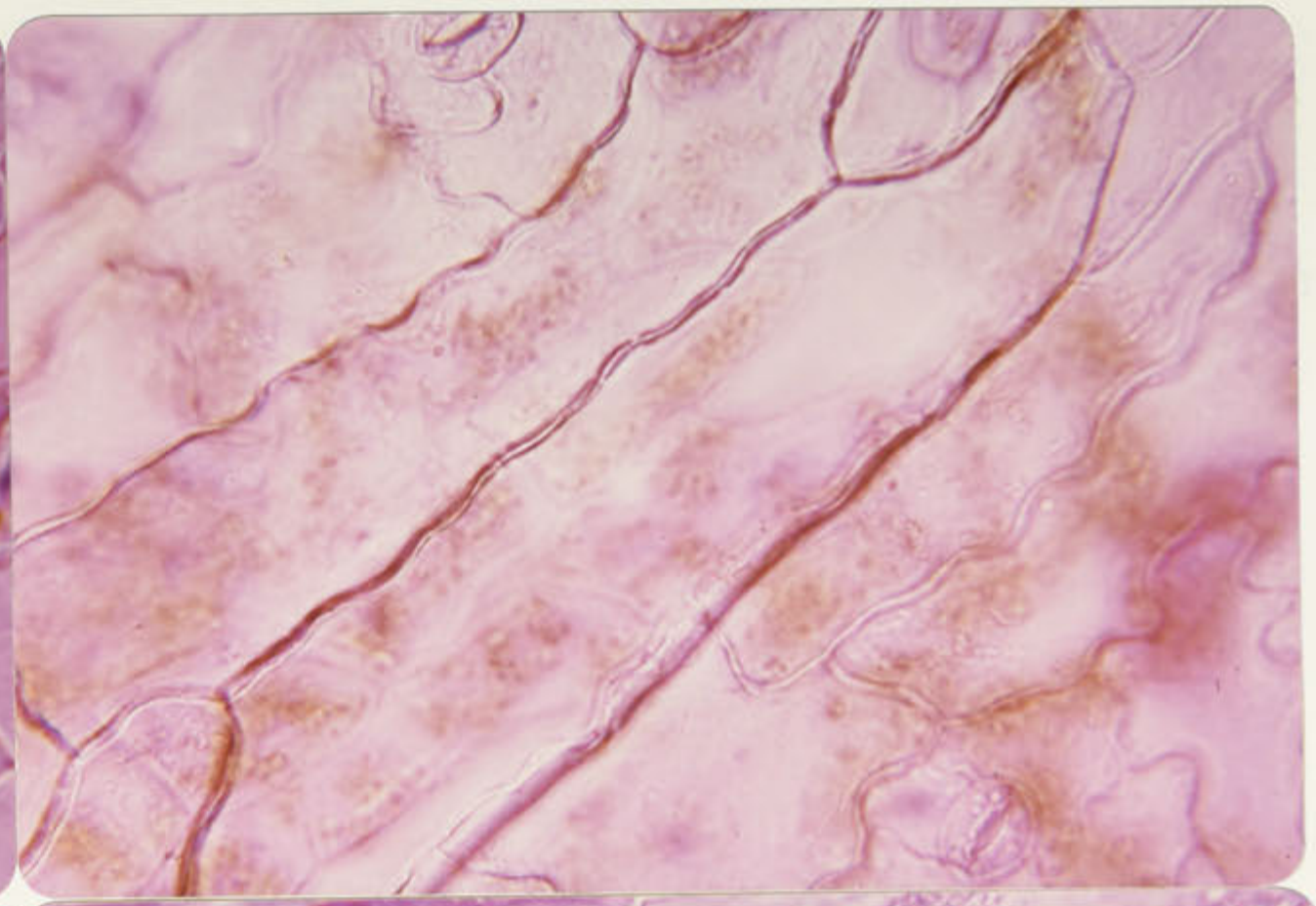
5.4. DISCUSSION

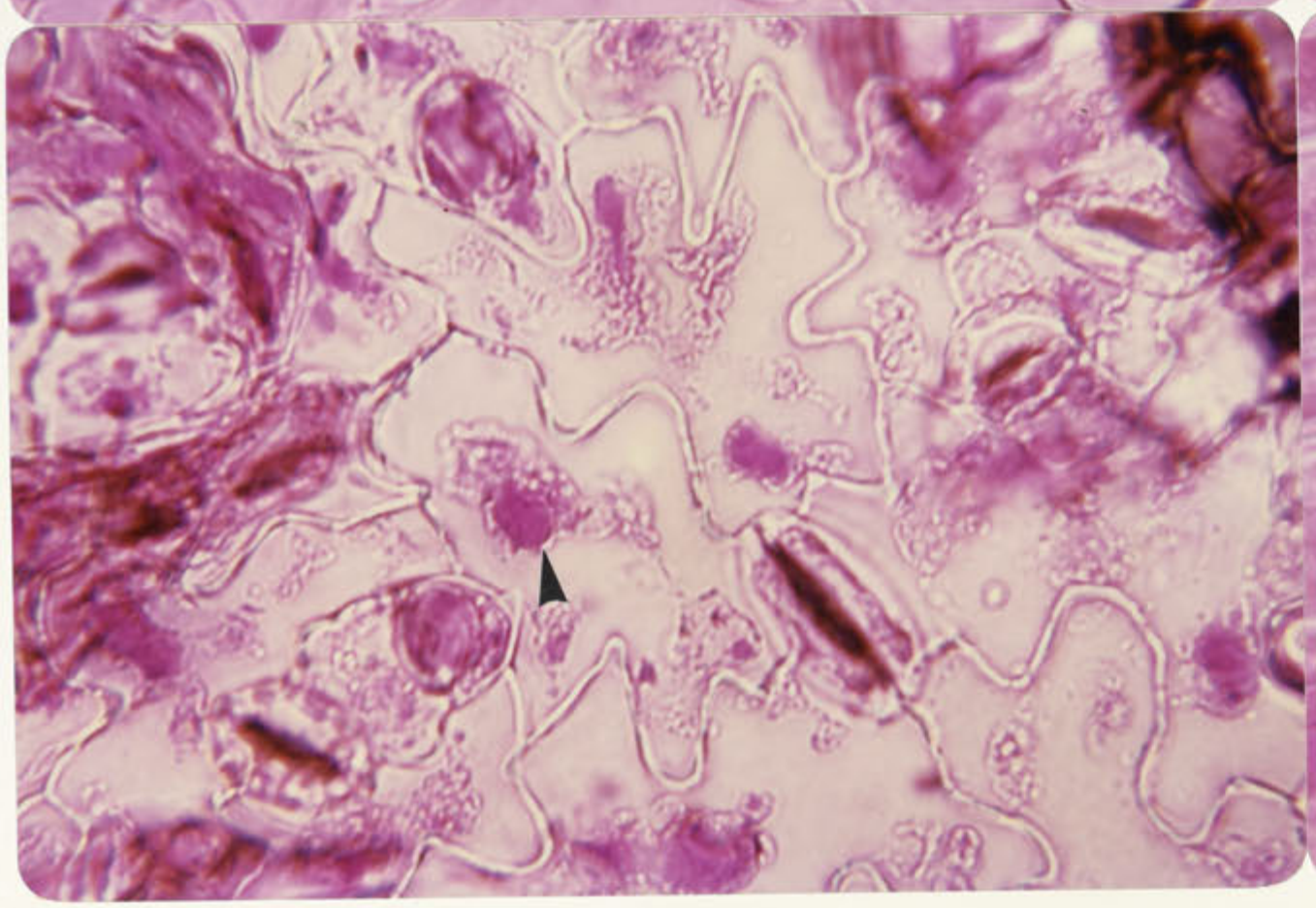
The establishment of cell suspension cultures of different *Brassica* sp. was not possible. Although callus was induced in all the species tested this was of poor quality and had a very slow growth rate. Probably, in some cases, it could have been induced to regenerate plants but never provided the fast manageable material, that would be best for a transformation system. From all the species tested the rapeseed cv. Turret was the most satisfactory but still 5 to 6 weeks elapsed between treatments before differences could be meaningfully evaluated. Attempts to obtain suspension cultures were unsuccessful.

With protoplasts the situation was similar. Good protoplasts were generally isolated but rarely or never divided in any media. To expect any success in a transformation system a reliable and fast

Fig. 41. Epidermal strip from leaves of turnip (*Brassica rapa*) from healthy plants (left hand side) and plants infected with CaMV (right hand side). The epidermal strip have been stained with 1% phloxine for 1 min and destained with distilled water. The arrows indicate the inclusion bodies formed by CaMV in the cytoplasm of the cells.







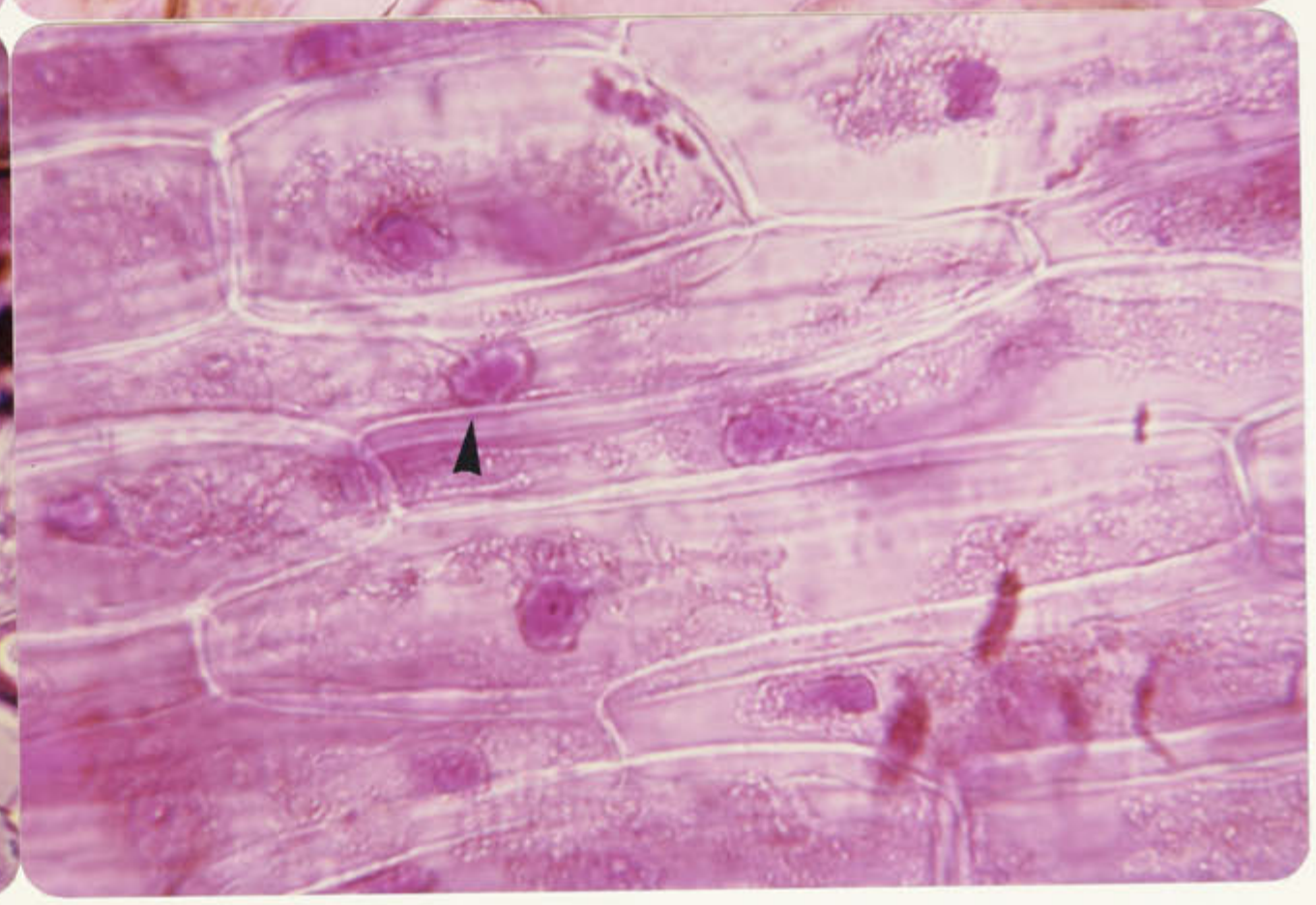
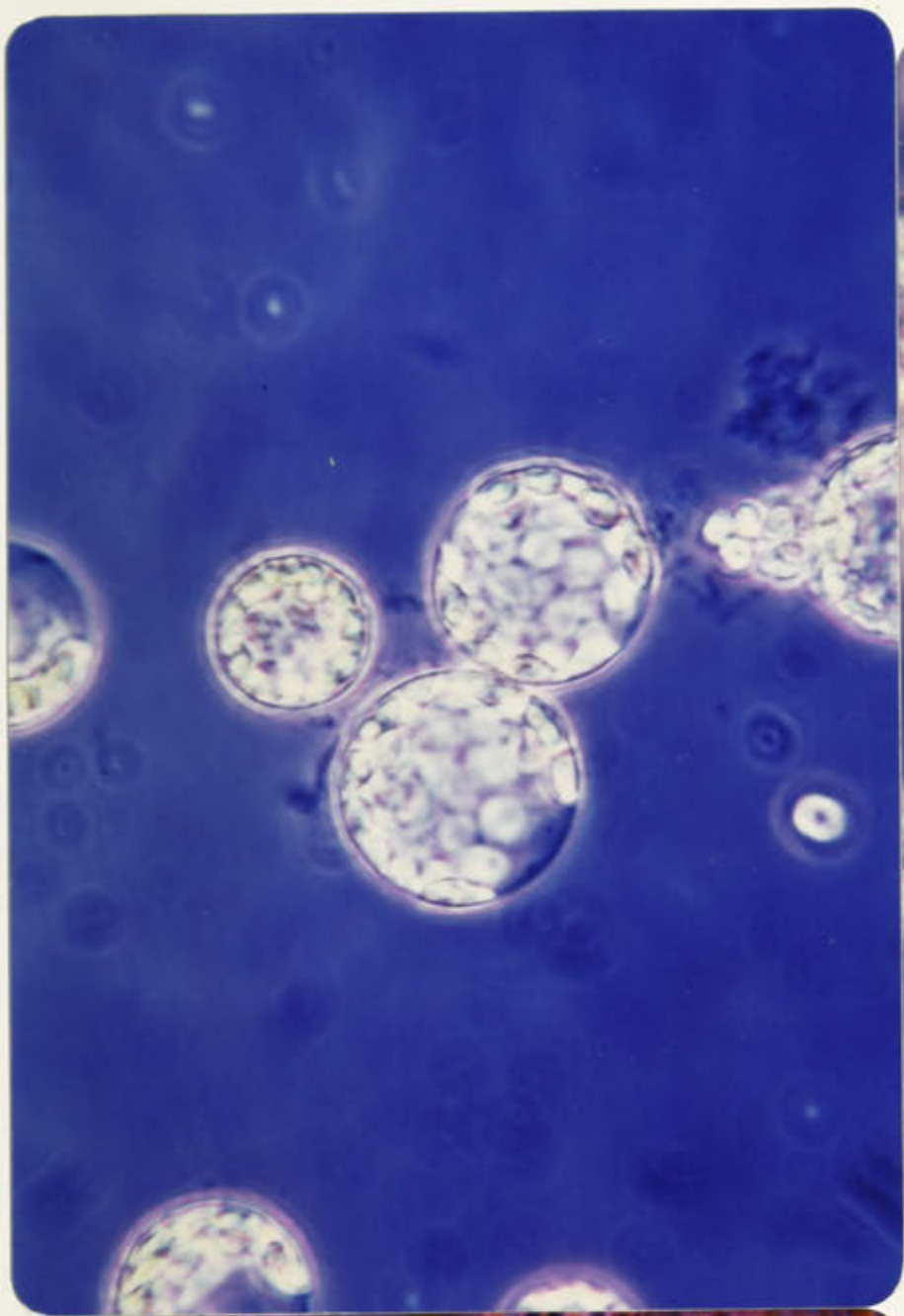
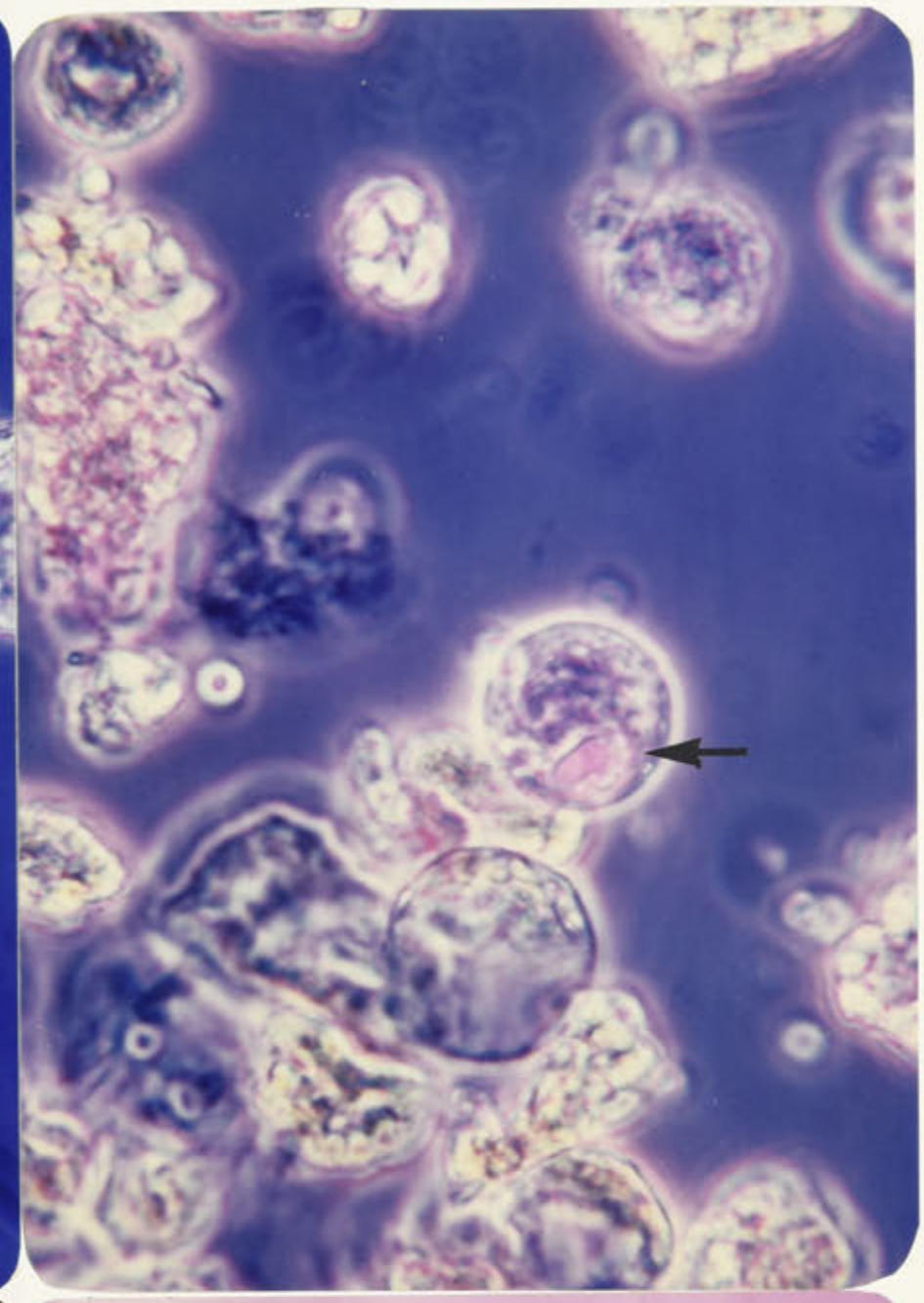
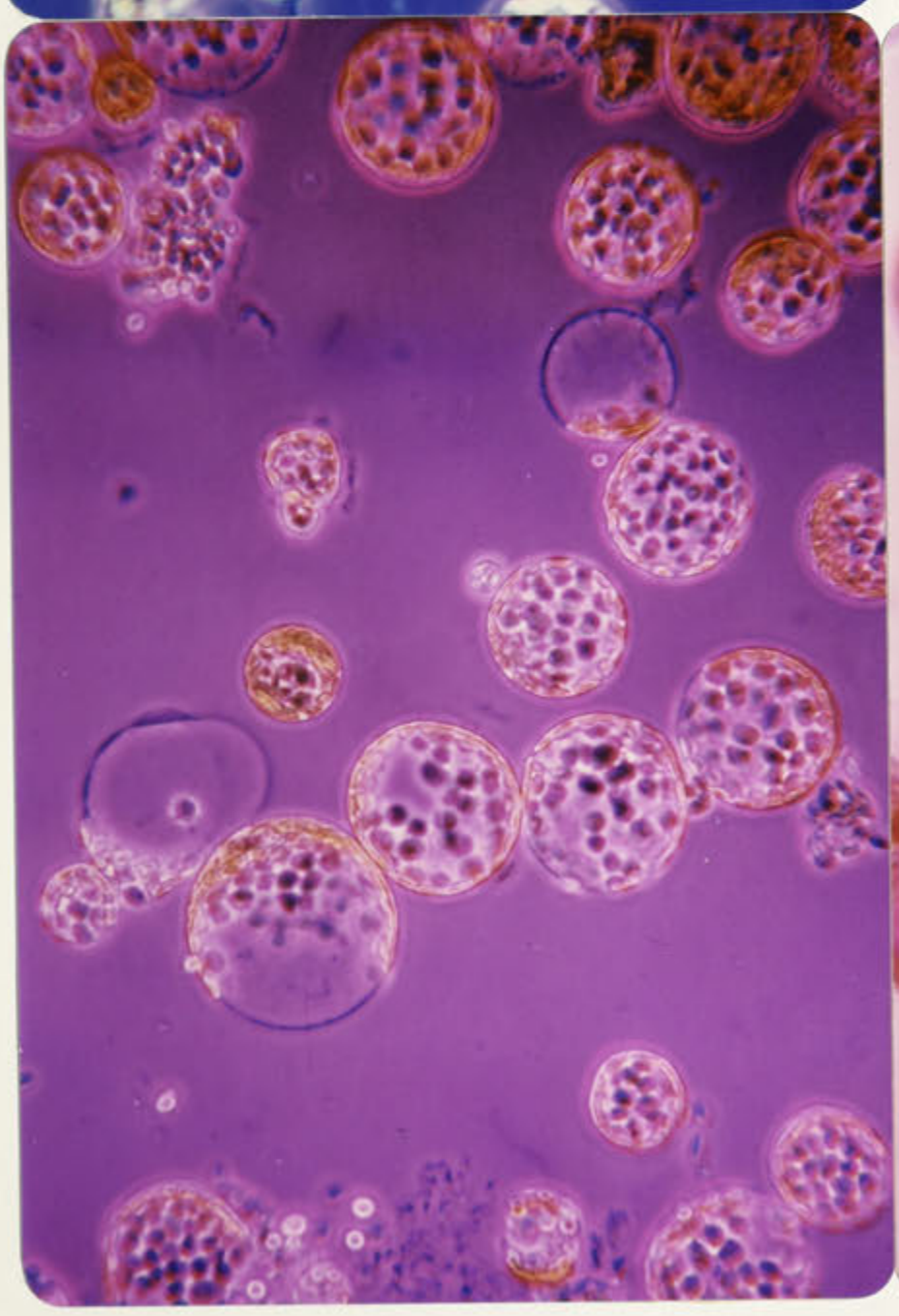
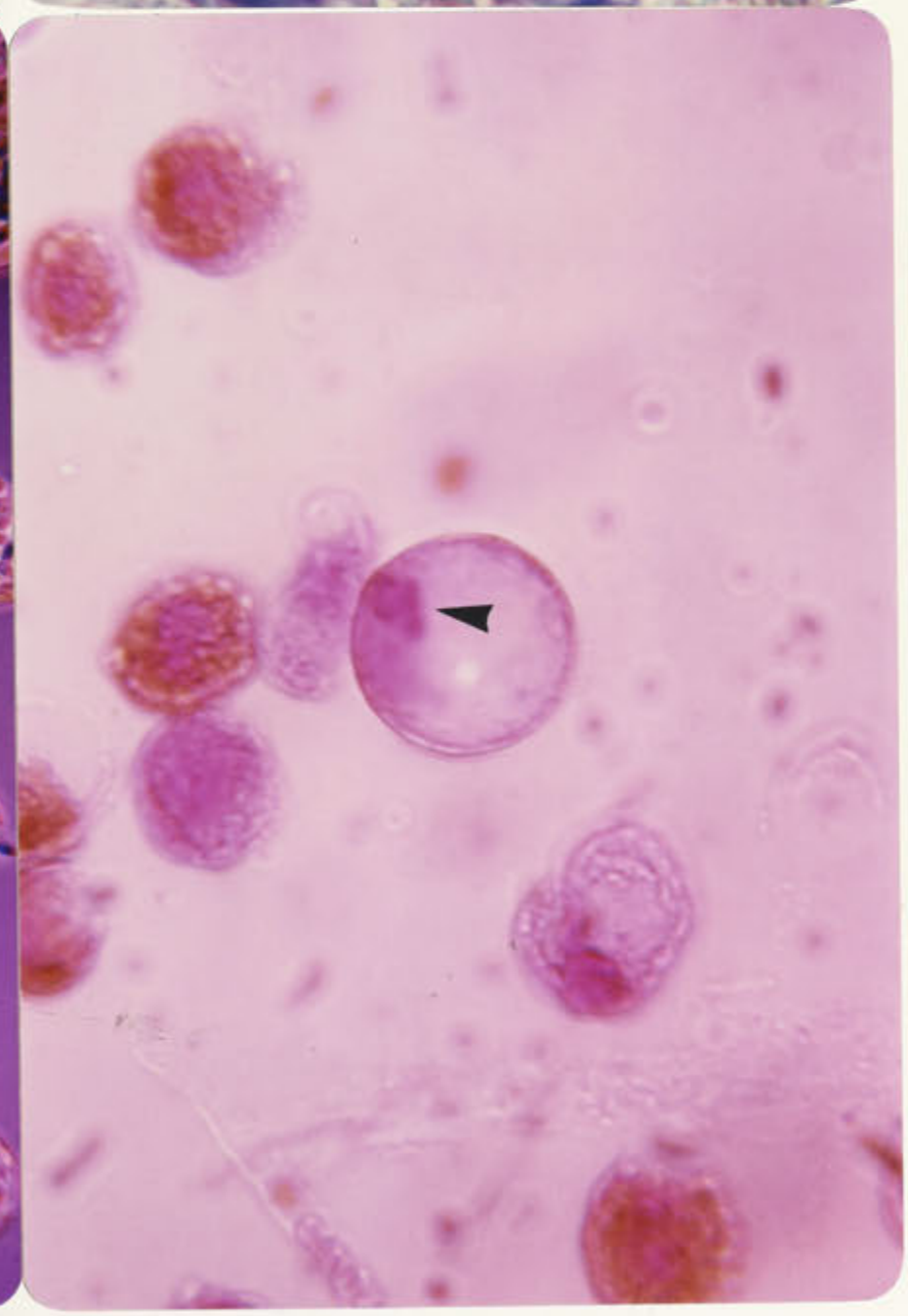


Fig. 42. Protoplasts obtained from leaves of turnip (*Brassica rapa*). The figures on the left hand side correspond to protoplast obtained from healthy plants. The figures on the right hand side correspond to protoplasts obtained from leaves infected with CaMV. The protoplasts have been stained with 1% phloxine in 0.5 M mannitol for 1 min and rinsed repeatedly with protoplast culture medium devoid of growth regulators. The arrows indicate inclusion bodies formed by CaMV in the cytoplasm of cells of infected plants.









rate of multiplication of protoplasts such as occurs with tobacco is essential.

The failures to obtain suitable cultures of *Brassica* cells induced me to look for alternative hosts. *Nicotiana* sp. in general, have been extensively used in tissue culture because they are extremely amenable to cell culture growth, plant regeneration and protoplast isolation and culture (Carlson *et al.*, 1972; Melcher, 1977; Kao, 1977; Smith *et al.*, 1976).

N. clevelandii has been reported as a host of CaMV (Hill and Campbell, 1968) and when tested, its cells grew very readily in culture. The principal remaining problem is that none of the strains of CaMV used in this study infect *N. clevelandii*. If CaMV strains that infect this host are obtained, this might be a good system for transformation. Studies in cell cultures of other species of *Nicotiana* could provide a useful background in developing selection systems to enhance the recovery of transformants.

Among the crucifers, *Arabidopsis thaliana* had been reported to grow reasonably readily in culture (Negrutiu, 1976) and being a crucifer might prove to be a host for CaMV. This was confirmed by infecting several races of *Arabidopsis* with the three strains of CaMV available. So far *A. thaliana* provides excellent cell cultures in which to establish a selection system. Protoplasts can also be readily isolated but they are proving recalcitrant to division in culture (W.R. Scowcroft - personal communication).

Every attempt described failed to develop satisfactory cultures from protoplast derived from plants which are (crucifers) or might be (*N. clevelandii*) hosts of CaMV although protoplasts of *N. clevelandii* were not adequately tested in culture since CaMV did not multiply in the plant.

Such cultures play a vital role in the plant transformation strategy outlined earlier.

Should the strategy be changed or should efforts to develop workable protoplast cultures continue? Given the nature of the progress in tissue culture research (stepwise advances due often to chance discovery of suitable media) it would seem that much trial and error is warranted before the possibility of success can be excluded.

The research reported in this chapter is, thus, seen as a minor attack on the problem, in no way permitting such a conclusion. Further attempts to develop protoplast cultures for infection by CaMV would seem justified, and further attention ought to be given to *N. clevelandii* and *A. thaliana*.

CHAPTER 6

PRESENCE AND NATURE OF NUCLEASES IN PLANT EXTRACT AND IN COMMERCIAL ENZYME PREPARATIONS USED TO PREPARE PROTOPLASTS FROM PLANT TISSUES OR CULTURED CELLS.

6.1. INTRODUCTION

Protoplasts are known to absorb macromolecules such as viral particles and viral RNA (Vasil, 1976). The absorption and maintenance of DNA has also been reported (Susuki and Takebe, 1976; 1978) but has proved to be more difficult to achieve. One reason for this might be the presence of nucleases in the plants, or in the cell cultures which could degrade the DNA molecules. Nuclease activity is affected by several factors including pH, availability of cations (Mg^{++}, Zn^{++}, Ca^{++}) and temperature. In order to optimize DNA uptake, a monitoring system to detect the presence and nature of nucleases would be desirable.

The cell wall degrading enzymes used to produce protoplasts are crude extracts of soil fungi and other cellulose degrading micro-organisms. Two of the most extensively used enzyme preparations are Onozuka-R10 cellulase (All Japan Biochem. Co. Kinki Yakult) which is derived from *Trichoderma viride* and Driselase (Kyowa Hakko Kogyo) isolated from a Basidiomycete.

Driselase has been reported to be harmful to some cells, damaging the plasmalemma membrane causing protoplast lysis (Erikson, *et al.*, 1974). In a preliminary study John Langridge (personal communication) found that DNA was partly hydrolysed by cellulase solutions, as shown by changes in optical absorbance (A_{260}) after different incubation periods.

Although the method could detect nuclease activity, it was not sensitive enough to monitor DNA uptake experiments and gave no idea of the nature of the nucleases.

In this chapter a method is described which uses radioactively labelled DNA substrates to monitor nuclease activity. It assesses the nuclease activity by measuring the proportion of radioactivity (hydrolysed) in the supernatant after precipitating the DNA substrate. Whether the enzymes were endonucleases or exonucleases could be determined by comparing their effect on linear or circular double-stranded DNA molecules.

6.2. MATERIALS AND METHODS

6.2.1. *Preparation of plant extracts*

Leaves of turnip (*Brassica rapa* cv. Just Right) were blended with 10 ml of distilled water for 2 min. After filtering through cheese cloth the sap was centrifuged for 10 min. at 5000 rpm in a Sorval SS-34 rotor. The supernatant was filtered through a millipore filter (Type GS 0.22 µm diameter) and stored at 4°C in a sterile plastic tube.

6.2.2. *Enzymes solutions*

1% solution of commercial Driselase, (Kyowa Hakko Kogyo) and Onozuka cellulase R-10 were prepared in water and dialyzed overnight in the cold against several changes of distilled water. After sterilising the solutions by filtering through Millipore filters (Type HA 0.45 µm diameter) these were stored at 4°C in plastic tubes.

6.2.3. *Preparation of DNA substrates*

Two substrates were used:

(a) A radioactively labelled ^{3}H (d AT) polymer, kindly provided by Dr. John Langridge (20.500 cpm/µg).

(b) Radioactively labelled plasmid pML 21. This plasmid was grown and extracted from its host *Escherichia coli* (PB 1141) as follows: Ten ml of bacterial culture were grown overnight at 37° in ML media (Bactotryptone 10 g/1, yeast extract 5 g/1, sodium chloride 5 g/1 of distilled water) plus 1% glucose, 330 mg/1 of deoxyadenosine and then added to 90 ml of the same media plus 7.5 mg of kanamycin. When the culture had an optical absorbance A_{600} of 0.5, chloramphenicol was added to give a final concentration of 180 µg/ml and the culture was shaken for 2 more hours at 37°C. Then 5 µCi/ml of [methyl-^{3}H]thymidine (1 mCi/ml; spec. activity 41 Ci/mmol) was added and the culture incubated overnight. The cells were collected by centrifuging at 10000 rpm for 10 min. washed with 10 ml of 2 x TES buffer (30 mM Tris, 2.5 mM EDTA, 50 mM NaCl, pH 8.0) and centrifuged again. Finally the culture was resuspended in 25% sucrose in 0.05 M Tris (pH 8.0) plus 2 x 10^{-5} M n-dodecylamine to give 4 ml final volume and transferred to a small flask kept at 0-4°C and 0.2 ml of fresh lysozyme (10 mg/ml) was added and gently swirled. After 10 min. 0.4 ml of cold 0.25 M EDTA (pH 8.0) was added and the mixture was dispensed drop by drop into 6 ml of lytic mix (0.3% Triton X-100, 5 mM Tris, 60 mM EDTA, pH 8.0) while rotating the flask at 50 rpm on ice. After rotating for 20 min the culture was centrifuged in the cold at 18,000 rpm for 20 min. and NaCl was added to the supernatant to give a final concentration of 0.5 M, then polyethylene glycol was added to give a concentration of 10% and the

mixture stirred in the cold until dissolved and the preparation incubated at 4°C overnight. The mixture was then centrifuged at 3000 rpm for 5 min. The sediment was resuspended in 2 ml TES plus sodium diethylpyrocarbonate (0.2%) and centrifuged at 10000 rpm for 10 min. The supernatant was collected and centrifuged in a CsCl ethidium bromide density gradient (refractive index = 1.3890) to equilibrium at 44,000 rpm for 48 hrs. Under ultra violet light two bands could be seen in the middle of the gradient (Fig. 43). The top band was chromosomal DNA and the bottom band was the pML 21 plasmid.

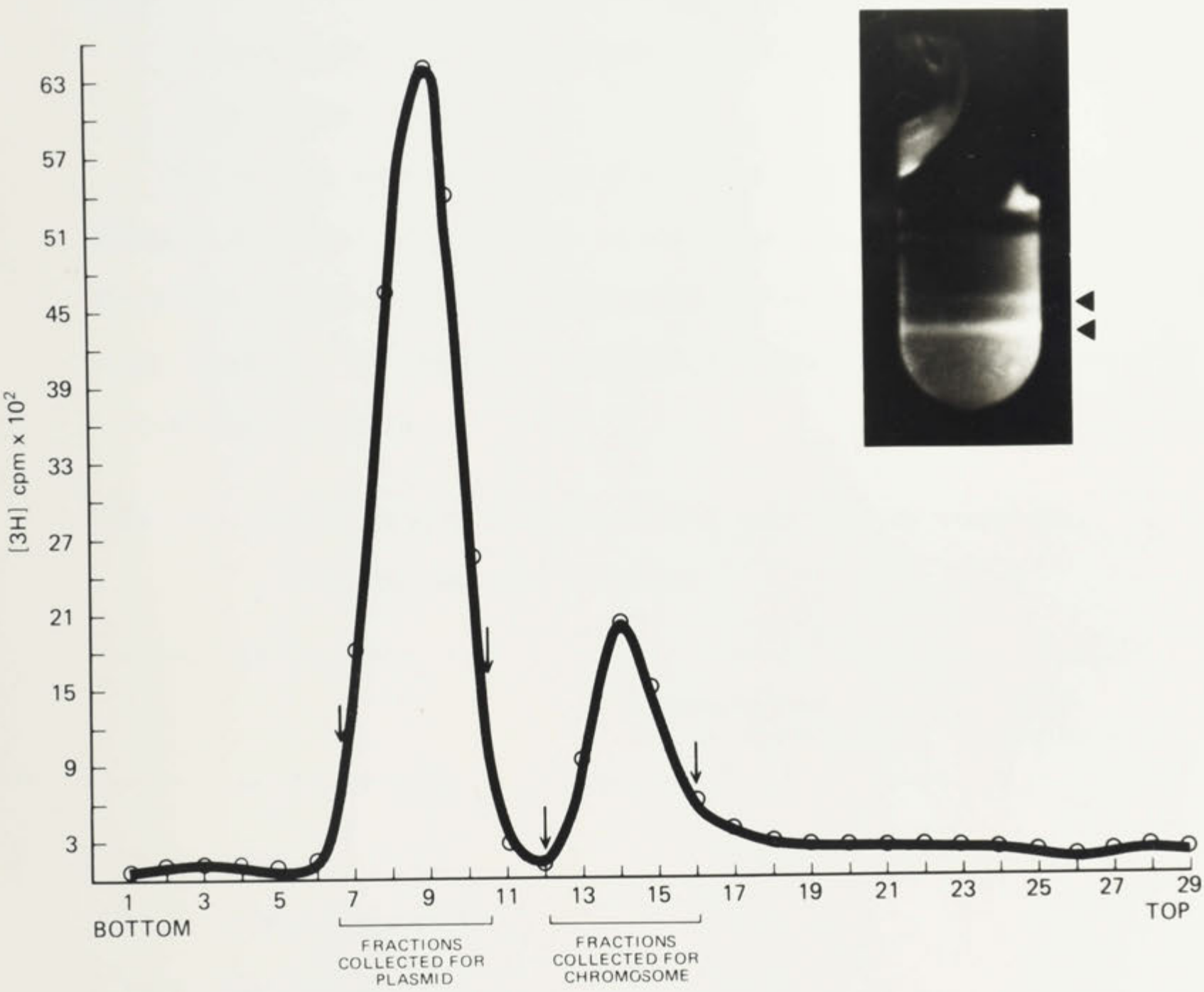
The gradient was collected in five drop fractions and the radioactivity of 10 µl every third fraction was counted (Fig. 43). Fractions 14-28 contained the plasmid and were pooled and fractions 35-45 the chromosomal DNA and were also pooled. After extracting the ethidium bromide with isoamylalcohol and ether the samples were dialyzed overnight against 10 mM Tris 1 mM EDTA buffer.

By this method 15.5 µg of chromosomal DNA (6,200 cpm/µg) were recovered and 58.1 µg of pML 21 plasmid (4,000 cpm/µg).

6.2.4. *Assessment of nuclease activity*

The labelled DNA substrates (1 µg, (^{3}H (d AT) polymer = 20,500 cpm, or 1.45 µg ^{3}H pML 21 plasmid = 6,000 cpm) were incubated with 1% of the enzyme solutions to be tested at 37°, after adding $MgCl_2$ to a final concentration 0.01 M in a volume adjusted to 150 µl with 0.1 M Tris buffer pH 8.0. After 90 min. calf thymus DNA was added as a carrier and precipitated by adding 0.35% cold perchloric acid and storing at 0° for 10 min. The precipitate was sedimented by centrifugation at 10,000 rpm for 5 min. and the radioactivity of the supernatant was measured in a scintillation counter after adding 8 ml of Triton

Fig. 43. Caesium chloride-ethidium bromide density gradient showing separation of ^{3}H labelled *E. coli* chromosomal DNA (top band) and plasmid pML21 DNA (bottom hand). The gradient was centrifuged to equilibrium at 44,000 rpm for 48 hrs in a Sorvall Ty65 rotor at 20°. The gradient was collected in five drop fractions and distribution of the radioactivity shown in the graph was determined by counting the radioactivity in 10 µl aliquots of each fraction. The arrows indicate the chromosomal and plasmid fractions.


[3H] cpm x 10^2
63
57
51
45
39
33
27
21
15
9
3
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
BOTTOM
TOP
FRACTIONS COLLECTED FOR PLASMID
FRACTIONS COLLECTED FOR CHROMOSOME

X-100 scintillation fluid (PPO 6 gr, POPOP 0.6 gr, Toluene 670 ml, Triton X-100 350 ml). As a control for endonuclease activity, commercial DNase I (Boehringer Mannheim) was added at a concentration of 5 µg and 10 µg. For exonuclease activity λ exonuclease 1 (10 µg) was used.

6.3. RESULTS

6.3.1. *Assessment of amounts of exonucleases capable of hydrolysing ^{3}H (d AT) polymer in different enzymes and plant extracts*

The results shown in Table 6.1 indicate that cellulase and plant extracts contained large amounts of nucleases that could hydrolyse the d (AT) polymer; 81.9% was hydrolysed by cellulase and 42.1% by plant extracts. By contrast, Driselase preparations hydrolysed only 5.5% of the substrate.

Table 6.1. Hydrolysis of [^{3}H]poly d (AT) substrate by Driselase, cellulase and plant extract.

	Radioactivity cpm in supernatant	% of hydrolysis
[^{3}H] d (AT)n	286	1.4
[^{3}H] d (AT)n + Driselase 1%	1135	5.5
[^{3}H] d (AT)n + cellulase 1 %	16783	81.9
[^{3}H] d (AT)n + plant extract 1%	8637	42.1

6.3.2. *Assessment of the nature of the nucleases present in the various test solutions*

The plasmid pML 21 being a closed covalent circular molecule is not attacked by exonucleases, but if endonucleases are present this would open the molecules rendering them susceptible to exonucleases.

The results shown in Table 6.2 indicate that the plasmid isolated was mainly in the circular form since it can only be substantially hydrolysed by endonucleases and not by exonucleases.

Cellulase and plant extracts hydrolysed the plasmid to a considerable extent indicating that they contained endonucleases, and possibly also exonucleases. No endonuclease activity was detected in driselase.

Table 6.2. Hydrolysis of closed covalent circular DNA molecules of PML 21 by endonuclease, exonuclease, plant extract and cell wall degrading enzymes.

	Radioactivity* cpm/1.45 µg of substrate (6000 cpm)	% hydrolysis of substrate
pML 21	120.9	2.0
pML 21 + DNase I (5 µg)	1211.8	20.2
pML 21 + DNase I (10 µg)	1924.1	32.1
pML 21 + Exonuclease (10 µg)	379.0	6.3
pML 21 + 0.6% plant extract	4609.2	76.8
pML 21 + 0.6% cellulase	4359.4	72.6
pML 21 + 0.6% driselase	125.6	2.1

* Average of 3 replicates.

6.4 DISCUSSION

The method reported in this chapter allows one to monitor the presence and nature of nucleases during DNA absorption experiments. This knowledge permits one to determine the conditions (i.e., pH, buffers to be used, temperature) that minimize hydrolysis of nucleic acids during uptake by protoplasts. It also could help determine which DNA to use, as in some systems exonuclease concentrations would be so great that linear DNA would be useless and only circular DNA could be used.

It is clear that during isolation and culture of protoplast from suspension cultures or from intact organs, contaminating nucleases, both exo- and endonucleases, are present.

In experiments designed to achieve uptake of naked DNA by plant protoplasts, the production of protoplasts by cellulase should be avoided. Driselase is obviously the cell wall degrading enzyme of choice.

The presence of high levels of both endo- and exo-nucleases in plant material is a problem that cannot be avoided or minimized by thorough washing as in the case with protoplasts. It is likely that plant cells continually produce nucleases. The use of naked DNA in uptake experiments demands that methods be developed which eliminate or substantially reduce nuclease activity of protoplasts. This might be achieved by use of low temperature incubation (1°C) or addition of sodium citrate (Slavik and Widhold, 1978) that have provided a certain degree of success.

Alternatively, the DNA could be encapsulated in vesicles, such as liposomes, which protect the entrapped DNA from degradation (Lurquin, 1979; Larkin and Scowcroft - personal communication).

CHAPTER 7

GENERAL DISCUSSION

7.1 CAULIFLOWER MOSAIC VIRUS - A POTENTIAL GENETIC VECTOR

A major aim of this work was to study the genome of CaMV and its organization as an essential first step in developing vectors, based on CaMV, for genetic transformation in plants.

The restriction endonuclease cleavage map of three different strains indicated that there was variation between strains at the DNA level. This variation could be caused by base substitution or small deletions. Moreover, heterogeneity or variation within strains was also observed and this might suggest that the replicative mechanism of the virus might not reject recombinant viral DNA molecules carrying fragments of foreign DNA.

Comparative studies of the three genomes showed that one part of the genome is particularly variable, namely the 1.2 Eco R1 fragment. Because this region appears to tolerate genetic variability, it is possibly the most appropriate region in which to introduce foreign DNA. If base substitutions and/or small deletions, such as that detected in the strain CM4-184 (Hull and Howell, 1978), occur naturally in this region without affecting the replication of the virus, it is possible that foreign DNA might also be accepted within this region.

Other results obtained in this work with cloned CaMV were less encouraging. The inability of cloned CaMV DNA to infect plants might mean:

(a) The viral DNA cannot be replicated in the plant.

(b) The viral DNA is replicated but is no longer able to cause symptoms in the hosts.

If the first hypothesis is correct, this might be due to loss or reorganization of genetic information required for replication of CaMV. An attempt was made to determine whether loss of infectivity of CaMV, cloned using the Bam I site, was due to obliteration of single-stranded gaps present in the genome.

There was an association between filling of the single-stranded gaps and loss of infectivity, but it is premature to ascribe a causal relationship between these two phenomena. Szeto *et al.* (1977) had earlier reported the loss of infectivity of CaMV DNA which had been cloned using the Sal I site. However, recent unpublished evidence (Genome Organization and Expression in Plants, Edinburgh, Scotland, 1979 - R. Appels, personal communication) indicates success in conserving infectivity of CaMV, cloned using the same Sal I site as that used by Szeto *et al.* (1977). If this is confirmed, further work in building recombinant molecules seems appropriate.

It would also be interesting to determine why the location of the cleavage site used to insert the viral molecule into the bacterial plasmid DNA influences its subsequent infectivity. In section 4.5.3 of this thesis several hypothesis have been advanced on this subject. A comparative study on the infectivity of cloned CaMV using sites other than Bam I and Sal I, e.g., Xho I, which is located at an intermediate position between the Bam I and Sal I sites could help in understanding this phenomenon.

If the second hypothesis is correct, and the CaMV is replicating in the host but no longer causes symptoms, then as a vector a CaMV without single-stranded nicks would be safer in a biohazard context. In this case, detection of virus replication would have to rely on more sophisticated tests than symptoms.

It is possible that gene products coded for by CaMV might be readily detectable. Meagher *et al.* (1977), for example, examined the synthesis of viral polypeptides induced by fragments of CaMV DNA contained in recombinant plasmid. CaMV DNA fragments directed the synthesis of high levels of three polypeptides, which were synthesised entirely from within the cloned virus DNA fragments, irrespective of the mode of their insertion into the plasmid. This was determined by using three different plasmids (pMB9, pRM39 and pRM51) and in each case polypeptides of the same electrophoretic mobility were recovered. This was interpreted as strong evidence that there is a degree of transcriptional and translational fidelity of plant virus DNA in bacteria. However, none of the polypeptides showed any detectable similarity to the virion proteins (Meagher *et al.*, 1977).

If CaMV is to be used as a vector a knowledge of its replication mechanism is essential. So far, there is little information (Fujisawa *et al.*, 1976; Favali *et al.*, 1973). Howell and Hull (1978) described a plant protoplast system in which replication could be studied. They used turnip protoplasts, but their system did not permit protoplast division and bacterial contamination was a problem. The time required for the appearance of mature CaMV particles was quite slow; 4-5 days. Also it was found that viral specific RNA preceded the production of mature virions, but no evidence for sequential production of stable RNA transcripts was found. The viral RNA constituted a small fraction of the total stable RNA in the infected protoplast and was transcribed from only one DNA strand of the CaMV genome, namely the one having only one single-stranded nick (Strand X in Fig. 34). The RNA transcripts may have been messenger RNA (Howell and Hull, 1978) but no evidence of translation was presented.

An appropriate protoplast-cell culture system would greatly assist in the study of CaMV, its genetic make-up and its multiplication mechanism. Natural host species of CaMV have in general proven to be recalcitrant in culture, and this is particularly true of protoplast culture. There are reports that rape protoplast are amenable to culture (Kartha *et al.*, 1974) and a recent report by Gleba and Hoffman (1978) indicates that *Arabidopsis thaliana*, an alternative host for CaMV, can be cultured with low efficiency. Unpublished results by Brock, Larkin and Scowcroft (personal communication) indicate that culture of *Arabidopsis* protoplasts is very difficult.

It is also possible that non-natural host protoplast systems may provide a biological *"in vitro"* system for examining the replication of CaMV. The excellent protoplast culture systems developed for several species of *Nicotiana*, carrot and other cell suspensions should be examined as support systems for the replication of CaMV.

7.2 CONCLUDING REMARKS

Although CaMV has been an object of intense studies in past years, very little is known about the function and genetic organization of the genome, particularly on the replication of the virus. The primary interest in CaMV at the moment, is its possibility as a vector, and this resides in its replicon, to provide a sequence which is recognized by plant DNA polymerases.

In plant viruses, multiplication of viruses is usually assessed by production of symptoms in test plants, and this criterion has been applied to CaMV. But is infectivity in a pathological sense, essential for a vector? A vector containing all or part of CaMV may replicate in an appropriate host but it need not develop symptoms. Because of lack of other measures, infectivity or symptom development

is a means of monitoring multiplication. A reliable local lesion system used under appropriate conditions and statistical analysis can be utilized to estimate viral concentrations and thus monitor multiplication. But if symptoms are not produced, what is needed are other ways of detecting replication of CaMV or vectors containing part of the viral genome. Possibly specific and very sensitive serological techniques can be developed - although serology has not proven to be very effective with CaMV (Shepherd, 1979) in natural infections. Alternatively, procedures for *in situ* hybridization using radioactively labelled probes can be developed. In a protoplast-cell culture system this could be visualized considering all the technological advances already routinely used with animal cells. Also transformation systems can be used to advantage. Langridge (1978) reported a refined *E. coli* transformation system that can detect as little as 0.00004 µg of transforming DNA. Any of these, or other monitoring systems, need to be developed as an essential feature of a plant transformation system - symptomatology *per se* is inadequate. Moreover, replication of a vector without concommitant disease symptoms would be a decided advantage from the biohazard point of view.

Because of its relative uniqueness among plant viruses, i.e., double-stranded genomic DNA, an understanding of the molecular biology of CaMV has intrinsic value. The fact the DNA can be more easily handled and analysed than RNA might offer some advantages in studying phenomena common to plant viruses. CaMV might become a tool in the study of cross-protection between viruses, host specificity between others. It also could be valuable in comparative studies relative to bacterial DNA phages and or animal DNA viruses, to see if the physiology of the host can drastically influence the essential mechanisms of the virus.

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